May 28, 2017

Jean Claude Juncker
President, European Commission
European Commission
Rue de la Loi, 200
1049 Brussels
Belgium

By email only
(Cc to Jyrki Katainen, EC Vice President for Jobs, Growth, Investment and
Competitiveness; Vytenis Andriukaitis, EU Commisioner for Food Safety and
Health; Michael Flüh, DG SANTE; Bernhard Url, Executive Director, EFSA;
Giovanni La Via, Chair, ENVI Committee; EFSA Panel on Plant Protection
Products and their Residues; Andreas Hensel, President, BFR; Chris Wild,
Director, IARC; Wendy Cleland-Hamnett, Acting Associate Director, US EPA Office
of Chemical Safety and Pollution Prevention, Jose Tarazona, Pesticides Unit,
EFSA)

Open letter: Review of the Carcinogenicity of Glyphosate by EChA, EFSA and BfR

Dear President Juncker,

> **Executive Summary:** The European Food Safety Agency (EFSA) and the
> European Chemical Agency (EChA) have completed their assessments of the
> carcinogenic potential of glyphosate and concluded that the evidence does
> not support a classification for glyphosate. The raw data for the animal
> cancer studies for glyphosate have been released, and a reanalysis of these
> data show eight instances where significant increases in tumor response
> following glyphosate exposure were not included in the assessment by either
> EFSA or EChA. This suggests that the evaluations applied to the glyphosate
> data are scientifically flawed, and any decisions derived from these
> evaluations will fail to protect public health. I ask that the evaluations by
> both EFSA and EChA be repeated for all toxicological endpoints and the data
> underlying these evaluations be publicly released.

On November 27, 2015, my colleagues and I wrote to Commissioner
Andriukaitis[1] regarding the European Food Safety Agency (EFSA) and German
Federal Institute for Risk Assessment (BfR) reviews of glyphosate. At the time,
we had serious concerns regarding the scientific evaluation in the BfR
Addendum[2] and believed it was misleading with regard to the potential for
glyphosate to cause cancer in humans. On 13 January, 2016, we received a
response[3] from Dr. Bernhard Url, Director of EFSA. Since that time, both EFSA[4]
and the European Chemical Agency (EChA) have completed their carcinogenic
hazard evaluations for glyphosate and have concluded that the evidence does not
support a classification for glyphosate.

I continue to have serious concerns about the scientific quality of the evaluations
by both EFSA and EChA on a number of issues which were not adequately

addressed by Dr. Url in his response to the previous letter from me and my colleagues. These concerns will be reiterated at the end of this letter. There is, however, one topic I believe needs your immediate attention before a final decision is made regarding glyphosate re-authorisation. **Both EFSA and EChA (in their proposal of the dossier submitter[5]) failed to identify all statistically significant cancer findings in the chronic rodent carcinogenicity studies with glyphosate.**

On March 15, 2016, members of the European Parliament requested public access to the complete records of animal laboratory data from chronic carcinogenicity studies of glyphosate; these data were previously deemed to be confidential business information. The presence of this new information along with what was already available in the Supplemental Material from Greim et al. (2015)[6] allowed me to evaluate the data for any additional significant increases in tumor incidence that have not been reported in the evaluations by both EFSA and EChA. In these additional analyses, I found eight (8) significant increases in tumor incidence that do not appear in any of the publications or government evaluations presented by both EFSA and EChA. Table 1 summarizes those findings. Some of these tumors were also present in multiple other studies increasing the consistency of the findings across studies.

Transparency is an important aspect of the scientific process and I applaud EFSA for allowing limited access to the raw data from the animal studies of glyphosate. However, scientific rigor is required and the tumors identified in Table 1 may be interpreted as a failure by the agencies involved in these assessments to carefully review and analyze all of the available data before rendering a decision that there is no evidence that glyphosate is carcinogenic to humans. Some of these positive tumor findings may have been missed because two-sided tests[a] might have been used, but not all. In my opinion, one-sided tests[b] are more appropriate for public health evaluations.

As noted before, Monograph 112[7] from the International Agency for Research on Cancer (IARC) Monographs Programme evaluated the publicly accessible data for glyphosate and concluded that glyphosate is classifiable as probably carcinogenic to humans. IARC Working Groups routinely re-analyze some of the scientific data in the publications available to the working group to ensure that what is presented in a publication or technical document is correct. This is especially true for chronic studies of carcinogenicity in rodents. The IARC Working Group for Monograph 112 identified positive significant trends for tumors in two mouse carcinogenicity studies using the Cochran-Armitage linear trend test in proportions. Similarly, they identified a positive finding in one study in Sprague-Dawley rats. In their response to the IARC Monograph, the BfR re-evaluated some of the mouse data using this same statistical test.

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[a] A two-sided test addresses the question of whether glyphosate increased or decreased the tumor incidence. In an evaluation of this type, you are only interested in increases.
[b] A one-sided test addresses the question of whether glyphosate increased the tumor incidence

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Table 1: Eight additional tumor sites with significant (p<0.05) increases due to glyphosate exposure in the carcinogenicity studies cited by EFSA and EChA

Study Species	Tumor type Sex; Incidences	p-value[c] (one-sided)
Wood et al. (2009) CD-1 Mouse	Lung adenocarcinomas Males; 5/51, 5/51, 7/51, 11/51	0.028
Sugimoto et al. (1997) CD-1 Mouse	Hemangioma (any tissue) Female: 0/50, 0/50, 2/50, 5/50*	0.002
Atkinson et al. (1993) Sprague-Dawley Rat	Thyroid follicular cell adenomas and carcinomas Males; 0/50, 0/50, 0/50, 2/50, 2/49	0.034
Lankas (1981) Sprague-Dawley Rat	Thyroid c-cell Carcinomas Females; 1/47, 0/49, 2/50, 6/47	0.003
Enomoto (1997) Sprague-Dawley Rat	Kidney adenoma Male; 0/50, 0/50, 0/50, 4/50	0.004
Brammer (2001) Wistar Rat	Hepatocellular Adenoma Males; 0/53, 2/53, 0/53, 5/52*	0.008
Wood et al. (2009) Wistar Rat	Skin Keratocanthoma Males; 2/51, 3/51, 0/51, 6/51	0.030
	Mammary gland adenomas and adenocarcinomas Females; 2/51, 3/51, 1/51, 8/51*	0.007

* These groups have a significantly increased (p<0.05) incidence of tumors relative to the controls by the Fisher Exact Test in addition to a significantly positive trend test finding

Table 2 shows all of the statistically positive findings cited by EChA and an indication of whether these findings were known before the IARC Monograph. It appears, from my study of these documents, that BfR cited only four of these tumors prior to the IARC Monograph and identified an additional 9 positive findings after the IARC Monograph. I could find no comments in the EFSA Peer Review document[8] prior to the release of the IARC Monograph suggesting concern for these 9 positive tumor findings. Nor can I find any mention of the 8 positive tumor findings in Table 1. Thus, of the 21 positive tumor findings in Table 1 and Table 2, BfR, in their original submission, had only identified 20%.

In a recent interview on Euractiv.com[d], the EFSA spokesperson stated that *"EFSA and EU member states rely primarily on the original studies and the underlying raw data which they check themselves."* My review of the recently available data suggests this is not the case and that, again, several important positive findings have been missed. After the IARC Monograph review and after recognizing that there were other studies with positive results in these data that were not reported by the Glyphosate Task Force, it is difficult to understand why BfR, EFSA and EChA failed to re-evaluate all of the available data using an appropriate trend test.

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[c] The p-value presented here are from the exact Cochran-Armitage linear trend test in proportions.

[d] http://www.euractiv.com/section/agriculture-food/news/green-ngos-blame-monsanto-for-buying-science-to-save-glyphosate/

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Table 2: Tumor sites discussed in the draft CLH Report[5] which were identified either before or after the IARC Monograph[9]

Study Species, Duration	Tumor type, Sex	p-value[1] (HC)	IARC[2]	BfR[3]	Reason Not +[4]
Stout and Ruecker, (1990) Sprague-Dawley Rat 24 months	Pancreas islet-cell adenomas, Males[5]	0.147	yes	yes	a,b,c[6]
	Hepatocellular adenomas, Males	0.015	yes	no	b,c[6]
	Thyroid c-cell adenoma, Females	0.049	yes	no	b,c[6]
Lankas (1981) Sprague-Dawley Rat 26 months	Pancreas islet-cell tumors, Males[5]	0.315	yes	yes	a,b,c[6]
	Testes interstitial cell tumors, Males	0.009	yes	yes	a,c[6]
Wood et al. (2009) CD-1 Mice, 18 Months	Malignant Lymphoma, Male	0.007	no	no	c[7],d,e
Kumar (2001) Swiss Albino 18 Months	Malignant Lymphoma, Males[5]	0.096	no	no	c[7],d,e
	Malignant Lymphoma, Females	0.070	no	no	
Sugimoto (1997) CD-1 Mouse 18 Months	Malignant lymphoma, Males	0.016	no	no	c[7],d,e,f
	Renal adenoma, Males	0.062 (0.005)	no	no	c[7],f,g,h
	Hemangiosarcoma, Males	0.062 (0.004)[10]	no	no	c[7],f
Knezevich and Hogan (1983), CD-1 Mice 24 Months	Renal tumors, Males	0.065 (0.011)	yes	yes	c[7],d,e,f
Atkinson et al. (1993) CD-1 Mice, 24 Months	Hemangiosarcoma, Males	0.004 (0.001)	yes	no	c[7],f

[1] Exact Cochran-Armitage linear trend test in proportions, one-sided; (HC) is the probability of seeing the observed trend or greater assuming the mean of the historical control data for CD-1 mice from Giknis and Clifford (2000)[10] is correct (only applied to rare tumors)

[2] Identified in IARC Monograph

[3] Identified in BfR draft RAR prior to the IARC Monograph

[4] reasons cited by EChA for exclusion of the positive statistical finding: a-non clear dose-response; b-no progression to carcinoma; c-inconsistent across studies; d-trend test and pair-wise tests not consistent; e-historical controls with high incidence; f-in the range of the historical control data; g-tumors only at doses above 1000 mg/kg/day; h-no plausible mechanism

[5] the incidence counts for these studies in the draft EChA evaluation do not match the original pathology tables; p-values presented here relate to the original pathology counts

[6] comparing Sprague-Dawley rats with Wistar rats and studies at 26 months with studies at 24 months

[7] Comparing mice in 18-month studies with mice in 24-month studies

[10] No tumors were seen in 26 historical control groups so historical control response was set at the response that provides a 5% chance that we see 26 controls with no response – 0.0026

I am concerned that other areas of the EFSA review (e.g. reproductive toxicity and endocrine disruption) may have also received inadequate evaluations. Since the industry-supported scientific evidence is not available to external scientists, I am unable to evaluate these data and determine if there are positive findings

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that escaped detection. I encourage you to release these data for external analysis and review as well.

In summary, after numerous scientists from EFSA, from EChA, from BfR and from the Glyphosate Task Force have reviewed and evaluated this massive amount of data, there are still serious omissions in the way in which these data have been assessed and reported. I respectfully ask that the agencies involved in the evaluation of glyphosate conduct their own analyses of the tumor sites presented in Table 1 and amend the record of their decision as appropriate rather than simply ignoring these observations.

Even while I applaud the European Commission for a limited release of some of the information submitted by the registrants for glyphosate, it is still impossible for outside scientists to be fully confident in any reassessment of these studies. This is because important parts of the safety record are still sealed. While the raw data tables were made available upon a request by the members of the European Parliament, the materials and methods, analysis and discussion sections from these submissions are not available. These omissions make it impossible for outside scientists to judge the quality of the studies, the rigor of the methods used to analyze the data, or to determine if there are legitimate reasons in these discussions why the tumors identified in Table 1 were excluded.

Finally, in our previous letter, several major concerns were raised that have not been adequately addressed in the final assessments and should again be addressed appropriately. These are:
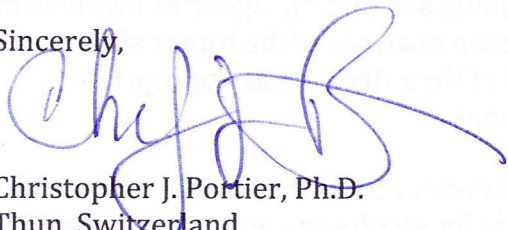
- the classification of the human evidence as "very limited" is not a valid characterization under the CLP guidelines and fails to properly address the strength of the available evidence;
- both EFSA and EChA dismissed positive findings because they fell inside of the range of the historical controls (this is an improper use of historical control evidence);
- both EFSA and EChA compared findings across different strains and different study durations to conclude that studies were inconsistent (this is not scientifically justifiable);
- both EFSA and EChA characterize the evidence for genotoxicity as negative, yet a careful review of the evidence released by EFSA and the open scientific literature suggest there are many guideline and non-guideline studies demonstrating genotoxicity.

I firmly support the principle that scientific evidence should be used to help guide societal decisions about health risks to humans. However, the individual scientific studies must be carefully summarized and reviewed if their findings are to serve as a true guidance. The glyphosate hazard classification appears to have been a good example of how lack of transparency regarding the scientific evidence that underlies important public health decisions can erode public trust and raise concerns. I respectfully request that you instruct the appropriate agencies to review the evidence submitted herein and ask that you refrain from making any decisions on glyphosate until these positive findings are included.

I also request that, in the interest of scientific transparency, EFSA should release all of the raw data in all areas of toxicology for all pesticides so scientists interested in repeating the evaluations by EFSA and EChA can do so.

Thank you for your time and I look forward to your response.

Sincerely,

Christopher J. Portier, Ph.D.
Thun, Switzerland
Former Director US National Center for Environmental Health
Former Director US Agency for Toxic Substances and Disease Registry
Former Associate Director, US National Institute of Environmental Health Sciences
Former Associate Director US National Toxicology Program
Fellow, American Statistical Association
Fellow, International Statistics Institute

Disclosures: The opinions expressed here and the analyses done to support those opinions are mine alone and were conducted without any compensation. In my capacity as a private consultant, I am an expert witness for a US law firm involved in glyphosate litigation. I also work part-time as a Senior Contributing Scientist for the Environmental Defense Fund (EDF) on issues not related to glyphosate or other pesticides.

References

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2.	(BfR)., G.F.I.f.R.A., *Final Addendum to the Renewal Assessment Report: Glyphosate*. October, 20154322.
3.	Url, B. *Response to Open Letter: Review of the Carcinogenicity of Glyphosate by EFSA and BfR*. 2016 1/13/2016; Available from: http://www.efsa.europa.eu/sites/default/files/EFSA_response_Prof_Portier.pdf.
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5.	German Federal Institute for Occupational Safety and Health, *Proposal for Harmonized Classification and Labeling: Glyphosate*, F.O.f. Chemicals, Editor. 2016: Dortmund, Germany.
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Nine studies showing glyphosate induce Parkinson disease by Brazil Netherland and Chinese scholars

巴西荷兰美国中国证实草甘膦诱发帕金森病九项科学证据
关键词：育儿 婴儿配方乳粉 化学浸出 正己烷 上海瑞金医院神经病学科

2001年以来，至少巴西、荷兰、美国与中国学者十项科学研究证实草甘膦对人类造成帕金森病，以及草甘膦造成帕金森病的细胞水平机制。这些研究中，国际《帕金森病相关失调》2011年发表中国学者陈生弟教授领衔团队《慢性职业性接触草甘膦造成帕金森病》与美国《神经病理学与畸形学》杂志2012年发表的接续研究《草甘膦通过凋亡和自我吞噬作用机制诱导细胞死亡》，无疑在国际上影响最大，国内外神经科学术界评价最高。上海交通大学医学院瑞金医院神经病学科与神经病学研究所陈生弟教授，是中国帕金森病诊断与治疗领域领先的专家。国内外学者这些研究综合在一起，构成草甘膦造成帕金森病科学上确凿无疑法律上有效证据链。农业部、中国疾控中心、国家食品药物监督管理总局的官员与公职专家，与"化学浸出"油产业，能不能否定巴西、美国与中国学者这五项科学研究的结果?当然可以，但是只能用更加严格的、科学公正长期的口服毒理学动物试验结果否定。

2001年以来，至少巴西、荷兰、美国与中国学者十项科学研究证实草甘膦对人类造成帕金森病，以及草甘膦造成帕金森病的细胞水平机制。

Since 2001, there are at least ten scientific studies by Brazilian, Netherland, American and Chinese scholars proving that glyphosate causes Parkinson's disease in humans, and the mechanism causes Parkinson's disease at cell level.

这些研究中，国际《帕金森病相关失调》2011年发表中国学者陈生弟教授领衔团队《慢性职业性接触草甘膦造成帕金森病》与美国《神

经病理学与畸形学》杂志2012年发表的接续研究《草甘膦通过凋亡和自我吞噬作用机制诱导细胞死亡》，无疑在国际上影响最大，国内外神经科学术界评价最高。

Among these studies, "Parkinsonism after chronic occupational exposure to glyphosate" published in 2011 by *Parkinsonism Related Disorder* by Prof. CHEN Sheng-di and his team, the their follow-up study "Glyphosate induced cell death through apoptotic and authophagic mechanisms" published in 2012 by *Neurotoxicology and Teratology*, no doubt . created greatest impact internationally, and received highest comments by the neurology science community in China and worldwide.

上海交通大学医学院瑞金医院神经病学科与神经病学研究所陈生弟教授，是中国帕金森病诊断与治疗领域领先的专家。

Prof. CHEN Sheng-di, Department of Neurology & Institute of Neurology, Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine, is a leading expert in diagnosis and treatment of Parkinson disease in China.

陈生弟教授与王刚医师领衔上海科学院上海生物科学研究所、健康科学研究所、肝细胞生物学神经组织退化疾病与重点实验室团队接续的《草甘膦通过凋亡和自我吞噬作用机制诱导细胞死亡》在细胞水平上揭示了草甘膦造成帕金森病的机理。

The follow-up study "Glyphosate induced cell death through apoptotic and authophagic mechanisms" by Prof. CHEN Sheng-di, together with Dr. Wang Gang and team at Lab of Neurodegenerative Diseases & key Laboratory of Stem Cell Biology, Institute of Health Science, Shanghai Institutes of Biological Sciences, Chinese Academy of Science, at cell level revealed the mechanism glyphosate caused Parkinson disease.

国内外学者这些研究综合在一起，构成草甘膦造成帕金森病科学

上确凿无疑法律上有效证据链。

These studies by overseas and Chinese scholars combined together, constitute the conclusive legally valid scientific evidence chain proving that glyphosate cause Parkinson's disease.

农业部、中国疾控中心、国家食品药物监督管理总局的官员与公职专家，与"化学浸出"油产业，能不能否定巴西、美国与中国学者这五项科学研究的结果?当然可以，但是只能用更加严格的、科学公正长期的口服毒理学动物试验结果否定。

The officials and official experts of the Ministry of Agriculture, the China CDC, the State Food & Drug Regulatory Administration Bureau, and the chemical solvent extraction food oil industry, can they deny the results of five scientific studies by Brazilian, American and Chinese scholars? Yes, they surely can, but only by the results of more rigid, scientific fair long-term oral feeding toxicology animal tests.

在他们组织这样的试验之前，他们只能够接受食品中草甘膦残留对人类造成帕金森病、对帕金森病发病率造成加剧作用的结论与后果。

Before they organize such tests, they can only accept the conclusion and consequences that glyphosate residues in food cause Parkinson to humans, and increase the incidence of Parkinson disease.

Scientific Evidence 1 (2001): Brazilian study: This 54-year-old man accidentally sprayed himself with the chemical agent glyphosate, a herbicide derived from the amino acid glycine. He developed disseminated skin lesions 6 hours after the accident. One month later, he developed a symmetrical parkinsonian syndrome. Two years after the initial exposure to glyphosate, magnetic

resonance imaging revealed hyperintense signal in the globus pallidus and substantia nigra, bilaterally, on T2-weighted images.

科学证据 1（2001）：巴西研究：这位 54 岁男性无意中对自己喷洒了草甘膦，氨基乙酸派生的除草剂。事故 6 小时后，他发生了弥散性皮肤损伤。一个月后，他发展了对称的帕金森病综合症。初次接触草甘膦后两年，磁共振成像，揭示 苍白球和塞美林氏神经节双边出现了 T2 加权像高信号。

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Scientific Evidence 2 (2003): Brazilian study: We report the brain magnetic resonance (MR) imaging abnormalities observed at the basal ganglia system of 5 patients (2 female and 3 male), who fulfilled the criteria of parkinsonism. For three patients, the diagnose of secondary parkinsonism was supported by clinical data: the first had the onset of the symptoms after the exposure to an herbicide (glyphosate).

科学证据2（2003）：巴西研究：我们报告对5位患者（两女三男）

在基底节观察到的脑磁共振图像异常，他们都满足了帕金森病的标准。… 第一位患者在接触除草剂（草甘膦）后出现了帕金森病的症状。

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Clínica Neurológica do Hospital das Clínicas da Faculdade de Medicina da Univesidade de São Paulo, São Paulo, SP, Brasil.
巴西医学大学临床医院神经学部

Scientific Evidence 3 (2007): American study: Previous studies based on limited exposure assessment have suggested that Parkinson's disease (PD) is associated with pesticide exposure. The authors used data obtained from licensed private pesticide applicators and spouses participating in the Agricultural Health Study to evaluate the relation of self-reported PD to pesticide exposure. This study suggests that exposure to certain pesticides may increase PD risk. Findings for specific chemicals may provide fruitful leads for further investigation. The full text of the study shows that the percentage of patients, who were later diagnosed with Parkinson disease, exposed to glyphosate herbicide was rather high among the pesticides,

indicating the association with glyphosate is rather high.

科学证据3（2007）：美国研究：基于有限接触评估的以前研究提议帕金森病与农药接触关联。作者们使用了自《农业健康研究》中有证书的私人农药使用者及其配偶的数据来评价自己报告的帕金森病与农药接触之间的关系。… 该研究提议某些农药可能提高帕金森病的风险。对具体化学品的发现对进一步研究提供有成效的线索。接触农药后来患帕金森病中接触草甘膦者比例较高，表明与草甘膦关联性较高。

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Kamel F et al.，农业健康研究中农药接触与自己报告的帕金森病，美国流行病学杂志，2007年2月；165(4):364-74.上线发布日期：2006年11月20日

Abstract/摘要：http://www.ncbi.nlm.nih.gov/pubmed/17116648
Full text/全文：http://aje.oxfordjournals.org/content/165/4/364.full.pdf

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Scientific Evidence 4 (2007): Netherland study: Previous systematic reviews have indicated that pesticide exposure is possibly associated with Parkinson disease (PD). Review and meta-analysis of 46 cohort studies, case–control studies, and cross-sectional studies, since 1950 to November 2010, that specifically investigated PD or parkinsonism, published in English, French, German, or Dutch., concerning herbicide, insecticide, and fungicide

exposure shows sRR (summary relative risk) for exposure to fungicides did not indicate an association with PD (overall sRR = 0.99; 95% CI: 0.71, 1.40), in contrast with positive sRRs for exposure to herbicides (overall sRR = 1.40; 95% CI: 1.08, 1.81) and insecticides (overall sRR = 1.50; 95% CI: 1.07, 2.11). Glyphosate based herbicide is the most widely used herbicide. Conclusion： Overall summary risk estimates strongly suggest that exposure to pesticides, and to herbicides and/or insecticides in particular, increases the risk of developing PD.

科学证据4（2007）：荷兰研究：过去的系统性审视表明暴露农药可能与帕金森病关联。对1950年以来至2010年期间46项英文、法文、德文或荷兰文发表的，涉及除草剂、杀虫剂与杀真菌剂调查帕金森病（PD）或帕金森神经机能障碍的队列研究、病例对照研究和横断面研究进行队列分析的，表明暴露于杀虫剂的sRR（相对风险总结）与帕金森病不相关(总sRR = 0.99; 95% CI: 0.71, 1.40），与此相反，暴露于除草剂有正向sRR（总sRR = 1.40; 95% CI: 1.08, 1.81），暴露于杀虫剂也正向sRR（总sRR = 1.50; 95% CI: 1.07, 2.11）。草甘膦除草剂是最为广泛使用的除草剂。结论：总体汇总风险估计强烈提议，暴露于农药，特别除草剂和/或杀虫剂，增加发展帕金森病的风险。

Marianne van der Mark et al., Is Pesticide Use Related to Parkinson Disease? Some Clues to Heterogeneity in Study Results

http://ehp.niehs.nih.gov/1103881/

http://www.medscape.com/viewarticle/759918

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Scientific Evidence 5 (2011): Chinese study: Report a patient with parkinsonism following chronic occupational exposure to glyphosate. A previously healthy 44-year-old woman presented with rigidity, slowness and resting tremor in all four limbs with no impairment of short-term memory, after sustaining long term chemical exposure to glyphosate for 3 years as a worker in a chemical factory. The chemical plant produced a range of herbicides including: glyphosate, gibberellins, and dimethyl hydrogen phosphate; however, the patient worked exclusively in the glyphosate production division. She only wore basic protection such as gloves or a face mask for 50 h each week in the plant where glyphosate vapor was generated. She frequently felt weak. Two months before she came to our clinic, she had experienced severe dizziness and blurred vision. Physical examination revealed a parkinsonian syndrome. There was no known family history of neurological or other relevant disorders. The patient had consumed no other medications or herbal preparations before the onset of symptoms. No report of parkinsonism induced by glyphosate after occupational exposure has been published to date.

科学证据5（2011）：中国研究：报告一个慢性职业性接触草甘膦续后患帕金森病的患者情况。她44岁，原先身体健康，在一个化工厂作为工人持续三年接触草甘膦后，四肢存在刚直、慢性与静止性震颤，但是短期记忆没有损伤。这家化工厂生产一系列除草剂，包括草甘膦、赤霉素，与二甲基磷酸氢盐；然而，这位患者仅在草甘膦生产部门工

作。工作期间她仅戴手套或口罩这样的基本保护，在产生草甘膦气体的部门每周工作50小时。她经常感到虚弱。来我们医院前两个月，她感到严重头昏眼花、视觉模糊。身体检查揭示了帕金森病综合症。患者没有任何已知的神经学的或相关失调家族史。帕金森病症状开始前，患者没有获得任何其他医药治疗或者草药治疗。到目前为止，没有公开发表过任何慢性职业性接触草甘膦诱发帕金森病的任何报告。

Wang G, Fan XN, Tan YY, Cheng Q, Chen SD., Parkinsonism after chronic occupational exposure to glyphosate. Parkinsonism Relat Disord. 2011 Jul;17(6):486-7.

王刚、范小宁、谭YY、程Q、陈生弟，慢性职业性接触草甘膦造成帕金森病（震颤麻痹综合症），帕金森病相关失调，2011年7月，17(6):486-7.

http://www.ncbi.nlm.nih.gov/pubmed/21367645?report=abstract

More details/更多内容：

http://www.deepdyve.com/lp/elsevier/parkinsonism-after-chronic-occupational-exposure-to-glyphosate-wpE2EMQQ6q

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As a broad-spectrum herbicide employed to kill weeds, glyphosate (N-(phosphonomethyl) glycine) is typically either sprayed to be absorbed through the leaves, injected into the trunk, or applied to the stump of a tree, and is also used to control vegetation around transmission towers, pipelines, water drainage channels, public squares, and streets throughout

the world.
作为一种广谱除草剂，草甘膦在世界各地用来杀死野草，通常或者喷洒到叶子上以便吸收，注射到树干中，或者应用于树墩，还用来治理输电塔、管道、排水渠、公共广场与道路旁的植物。

In China, glyphosate is popularly used as a hypotoxic weed-killer in rural areas. Every year, there are reports of acute intoxication of glyphosate due to attempted suicide or error in usage among adults and children. [2] Symptoms in such cases are frequently reported as consisting of digestive tract dysfunction, circulatory and respiratory failure, and liver and kidney damage [1, 2].
在中国，草甘膦在农村作为一种弱毒性除草剂普遍使用。每年都有成年人与儿童故意自杀或者误服草甘膦造成急性中毒的报告。[2] 这样的事例中经常报告的症状包括消化系统失调、血液循环与呼吸系统衰竭，以及肝脏和肾脏损伤。[1,2]

Neurological involvement, in particular extrapyramidal symptoms and signs including limb rigidity and resting tremor has only been reported following a few isolated events rather than in the setting of chronic occupational exposure [3].
神经系统的参与，尤其是锥体外系症状与肢体刚直与静止性震颤，仅在个别孤立事件续后有所报告，但是一直没有慢性职业性接触背景下的这种报告[3]。

Here we report a patient with parkinsonism following chronic occupational exposure to glyphosate. A previously healthy 44-year-old woman presented with rigidity, slowness and resting tremor in all four limbs with no impairment of short-term memory, after sustaining long term chemical exposure to glyphosate for 3 years as a worker in a chemical factory. The chemical plant produced a range of herbicides including: glyphosate, gibberellins, and dimethyl hydrogen phosphate;

however, the patient worked exclusively in the glyphosate production division. She only wore basic protection such as gloves or a face mask for 50 h each week in the plant where glyphosate vapor was generated. She frequently felt weak. Two months before she came to our clinic, she had experienced severe dizziness and blurred vision.

这里我们报告一个慢性职业性接触草甘膦续后患帕金森病的患者情况。她44岁，原先身体健康，在一个化工厂作为公认持续三年接触草甘膦后，四肢存在刚直、慢性与静止性震颤，但是短期记忆没有损伤。这家化工厂生产一系列除草剂，包括草甘膦、赤霉素，与二甲基磷酸氢盐；然而，这位患者仅在草甘膦生产部门工作。工作期间她仅戴手套或口罩这样的基本保护，在产生草甘膦气体的部门每周工作50小时。她经常感到虚弱。来我们医院前两个月，她感到严重头昏眼花、视觉模糊。

After being diagnosed by the local doctor with cervical spondylosis, the patient received treatment with DAN-SHEN (salvia) injections for one week without any improvement.

当地医生诊断为颈椎病以后，给患者注射了一周丹参（鼠尾草），但是没有任何改善。

Physical examination revealed a parkinsonian syndrome. There was no known family history of neurological or other relevant disorders. The patient had consumed no other medications or herbal preparations before the onset of symptoms.

身体检查揭示了帕金森病综合症。患者没有任何已知的神经学的或相关失调家族史。帕金森病症状开始前，患者没有获得任何其他医药治疗或者草药治疗。

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No report of parkinsonism induced by glyphosate after occupational exposure has been published to date.

到目前为止，没有公开发表过任何慢性职业性接触草甘膦诱发帕金森

病的任何报告。

In 2001, Barbusa et al. reported a case resulting from spraying glyphosate in a garden without wearing protection [3]. The patient had acute skin lesions one week after the chemical exposure and displayed rigidity and slowness in all four limbs one month after the initial exposure. One year later, he developed a slow resting tremor in the left hand and arm, accompanied by impairment of short-term memory.

2001年，Barbusa et al.报告过在花园中喷洒草甘膦而没有戴任何防护面具的一个案例[3]。化学接触一周后，患者发生急性皮肤损害，而且，在初次接触草甘膦后一个月四肢显示刚直性与缓慢性。一年后，患者左手与左臂发展了慢性静止性震颤，同时出现短期记忆损伤。

Although our patient had similar extrapyramidal symptoms, she had neither skin lesions nor memory loss.

我们的患者，尽管存在类似的锥体外系症状，她既没有皮肤损伤，也没有记忆损伤。

In a series of experiments, glyphosate demonstrated a wide range of toxicities for enzymes such as cholinesterase, carboxylesterase, and glutathione S-transferase [4].

一系列的实验中，草甘膦显示对胆碱酯酶、羧酸酯酶，与谷胱苷肽S-转移酶这些酶造成广泛一系列毒性[4]。

Recently, a case of glyphosae-surfactant induced reversible encephalopathy was reported and it was suggested that glyphosate-surfactant could induce a prolonged but reversible encephalopathy suggestive of acute central nervous system toxicity different from previously reported symptoms and disorder including: nausea, vomiting, oral and abdominal pain, renal and hepatic impairment, and pulmonary edema [5].

不久前，报告了一项草甘膦—表面活性剂诱发的可逆性脑病案例，它提议草甘膦—表面活性剂能够诱发延长的但是可逆性脑部，提议存在着一种急性中枢神经毒性，其症状与以前报告的症状与失调有所不同：恶心、呕吐、口腔与腹部疼痛、肾脏与肝脏损伤，以及肺水肿[5]。

In these previous investigations [1, 3, 5], the neurotoxicity of glyphosate was suggested to be via an excitotoxic mechanism. Unfortunately, there are to date no confirmed studies of neurotoxicity focused on the relationship between dopaminergic neuro transmission and glyphosate in the literature.

以前的这些调查中 [1, 3, 5]，草甘膦的神经毒性被建议为通过一种兴奋性毒性机制。遗憾的是，到目前为止，科学文献中还没有聚焦于多巴胺能神经传递与草甘膦之间关系的确认研究。

Scientific Evidence 6 (2012): Chinese study: Herbicides have been recognized as the main environmental factor associated with human neurodegenerative disorders such as Parkinson's disease(PD). Previous studies indicated that the exposure to glyphosate, a widely used herbicide, is possibly linked to Parkinsonism, however the underlying mechanism remains unclear. We investigated the neurotoxic effects of glyphosate in differentiated PC12 cells and discovered that it inhibited viability of differentiated PC12 cells in dose-and time-dependent manners. Furthermore, the results showed that glyphosate induced cell death via autophagy pathways in addition to activating apoptotic pathways. Interestingly, deactivation of Beclin-1 gene attenuated both apoptosis and autophagy in glyphosate treated differentiated PC12 cells, suggesting that Beclin-1 gene is involved in the crosstalk between the two mechanisms.

科学证据6（2012）：中国研究：除草剂被承认是与人类帕金森病这样的神经组织退化性失调相关的主要环境因素。以前的研究表明，接触于广泛使用的草甘膦除草剂，可能与帕金森病（震颤性麻痹症）关联，然而，作为其基础的机制依然不清楚。我们在分化型PC12细胞中研究了草甘膦的毒害神经作用，发现草甘膦以剂量—时间依赖性方式抑制分化型PC12细胞的发育能力。此外，研究的结果表明，在激活凋亡通路之外，草甘膦还通过细胞自我吞噬作用同路诱发细胞的死亡。有意思的是，在草甘膦处理的分化型PC12细胞，钝化Beclin-1基因减弱细胞凋亡及其自我吞噬作用，提议Beclin-1基因涉入了这两个机制之间的串扰。

Ya-xing Gui, Xiao-ning Fan，Hong-mei Wang，Gang Wang，Sheng-di Chen，Glyphosate induced cell death through apoptotic and authophagic mechanisms，Neurotoxicology and Teratology,Volume 34, Issue 3, May–June 2012, Pages 344–349

桂雅星、范小宁，王红梅，王刚，陈生弟，草甘膦通过凋亡和authophagic机制诱导细胞死亡，《神经病理学与畸形学》杂志，34卷第3期，2012年5-6月， 344-349页

http://www.sciencedirect.com/science/article/pii/S0892036212000438
https://www.ncbi.nlm.nih.gov/pubmed/22504123

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Scientific Evidence 7 (2012): Chinese study: Glyphosate is a worldwide widely used herbicide. In China, glyphosate is widely used in rural areas as a low-toxicity herbicide. For a long time, acute toxicity cases caused by glyphosate is frequently reported, toxicity symptoms include digestive tract disorders, blood circulation disorder and respiratory failure, liver and kidney damage, etc. During recent years, nerve system damage, especially extrapyramidal signs and symptoms caused by glyphosate have been reported. We took the lead reporting internationally a case of occupational glyphosate women patients with a history of chronic exposure to glyphosate appearing with lethargic and static rigidity, tremor and Parkinson's symptoms.

科学证据7（2012）：中国研究：草甘膦(glyphosate)即N-(磷酰基甲基)甘氨酸,是一种在世界范围内被广泛使用的广谱除草剂.在我国,草甘膦作为一种低毒除草剂在农村被普遍使用.长期以来,草甘膦导致急性中毒的报道屡见不鲜,其中毒表现常为消化道功能紊乱,血液循环障碍和呼吸衰竭,肝、肾功能损害等.近年来,草甘膦慢性中毒引起的神经系统功能损害,特别是锥体外系症状和体征陆续被报道.我们曾率先在国际上报道了1例职业性草甘膦慢性接触史的女性患者出现强直、行动迟缓和静止 性震颤等帕金森症状。

桂雅星，王刚，陈生弟,草甘膦暴露与帕金森病，中华神经科杂志，2012, 45

GUI Ya-xing, WANG Gang, CHEN Sheng-di, Chinese Journal of Neurology, 2012,45

http://d.wanfangdata.com.cn/Periodical/zhsjk201211015

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Scientific Evidence 8 (2012): American study: Previous studies demonstrate a positive correlation between pesticide usage and Parkinson's disease (PD), which preferentially targets dopaminergic (DAergic) neurons. In order to examine the potential relationship between two common pesticides and specific neurodegeneration, we chronically (24 hours) or acutely (30 min) exposed two *Caenorhabditis elegans* (*C. elegans*) strains to varying concentrations (LC$_{25}$, LC$_{50}$ or LC$_{75}$) of TouchDown® (TD) as per cent active ingredient (glyphosate), or Mancozeb® (MZ) as per cent active ingredient (manganese/zinc ethylene-*bis*-dithiocarbamate). Furthermore, to more precisely model environmental exposure, worms were also exposed to TD for 30 min, followed by 30-min incubation with varying MZ concentrations. Analysis of the BZ555 worms indicated a statistically significant decrease (*$P<0.05$) in number of green pixels associated with DAergic neurons in both treatment paradigms (chronic and acute) when compared to CN. Taken together, our data suggest that exposure to TD and/or MZ promotes neurodegeneration in both GABAergic and DAergic neurons in the model organism *C. elegans*.

科学证据8（2012）：美国的研究：以前的研究表明农药使用与帕金森病之间存在正相关性，且以多巴胺能神经元(DAergic)为优先目标。为了审查两种普遍性的农药与特定神经退化之间的潜在关系，我们将两种秀丽隐杆线虫（*C. elegans*）慢性（24小时）或急性（30分钟）暴露于其不同浓度（LC$_{25}$, LC$_{50}$ or LC$_{75}$）活性物质（草甘膦）TouchDown®（TD）除草剂，或活性物质（代森锰锌）Mancozeb®

（MZ）杀菌剂。此外，为了更精确模拟环境性暴露，线虫还暴露于草甘膦TD除草剂30分钟，再与不同浓度代森锰锌MZ杀菌剂一起培育。对BZ555丝虫的分析表明，与对照CN相比，在慢性以及急性处理两种范例中，与DAergic神经元相关的绿色像素的数量均统计意义下降（*P<0.05）。综合考虑，我们的数据提议，暴露于草甘膦TD除草剂和/或代森锰锌MZ杀菌剂，均促进模型生物体秀丽隐杆线虫中GABAergic与DAergic神经元的神经退化。

Rekek Negga et al., Exposure to Glyphosate- and/or Mn/Zn-Ethylene-bis-Dithiocarbamate- containing Pesticides Leads to Degeneration of *γ-Aminobutyric Acid* and *Dopamine Neurons* in *Caenorhabditis elegans*, Neurotox Res. 2012 Apr; 21(3): 281–290.

Rekek Negga et al.，暴露于含草甘膦和/或代森锰锌的农药导致秀丽隐杆线虫中γ-氨基丁酸与多巴胺神经元的退化，神经毒性研究。2012年4月；21(3): 281–290.

http://www.ncbi.nlm.nih.gov/pubmed/21922334/

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Scientific Evidence 9 (2014): American study: Within the last 20 years there has been an alarming increase in serious illnesses in the US, along with a marked decrease in life expectancy (Bezruchka, 2012). The Centers for Disease Control and Prevention (CDC) estimates that the cost of diabetes and diabetes-related treatment was approximately $116 billion dollars in 2007. Estimated costs related to obesity were $147 billion in 2008 and cardiovascular diseases and stroke were $475.3 billion in 2009. Health care expenditures in the US totaled 2.2 trillion

dollars in 2007 (CDC, 2013a). The onset of serious illness is appearing in increasingly younger cohorts. The US leads the world in the increase in deaths due to neurological diseases between 1979-81 and 2004-06 for the 55-65 age group (Pritchard et al., 2013). These mental disorder deaths are more typical of the over 65 age group. There have been similar findings for obesity, asthma, behavior and learning problems, and chronic disease in children and young adults (Van Cleave et al., 2010). Type II diabetes in youth is being called an epidemic (Rosenbloom et al., 1999). The rate of chronic disease in the entire US population has been dramatically increasing with an estimated 25% of the US population suffering from multiple chronic diseases (Autoimmunity Research Foundation, 2012). These findings suggest environmental triggers rather than genetic or age-related causes. A huge increase in the incidence and prevalence of chronic diseases has been reported in the United States (US) over the last 20 years. Similar increases have been seen globally. The herbicide glyphosate was introduced in 1974 and its use is accelerating with the advent of herbicide-tolerant genetically engineered (GE) crops. Evidence is mounting that glyphosate interferes with many metabolic processes in plants and animals and glyphosate residues have been detected in both. Glyphosate disrupts the endocrine system and the balance of gut bacteria, it damages DNA and is a driver of mutations that lead to cancer. In the present study, US government databases were searched for GE crop data, glyphosate application data and disease epidemiological data. Correlation analyses were then performed on a total of 22 diseases in these time-series

data sets. The Pearson correlation coefficients are highly significant with Parkinson disease (R = 0.952).

科学证据9（2014）：美国的研究：过去二十年内，一系列严重疾病在美国令人警觉增加，在此同期，预期寿命也明显减少（Bezruchka, 2012）。美国疾控中心（CDC）预计糖尿病以及糖尿病相关治疗费用2007年达到大约1160亿美元。与肥胖症有关的治疗费用2008年达到1470亿美元，心血管疾病与中风治疗费用2009年达到4753亿美元。美国2007年医疗保健支出达到22000亿美元(CDC, 2013a)。严重疾病的发病越来越多出现在年轻人群。美国1979年-1981年与2004年-2006年期间55-65岁由于神经性疾病的死亡率领先世界（Pritchard et al., 2013）。这些精神疾病死亡在65岁以上人群中更为典型。儿童与青少年人中肥胖症、气喘、行为和学习问题与慢性疾病有类似的发现（Van Cleave et al., 2010）。美国年轻人中的乙型肝炎被称为流行病（Rosenbloom et al., 1999）。美国人口中慢性病发病率剧增，美国人口中预计25%的人遭受多种慢性疾病（Autoimmunity Research Foundation, 2012）。这些发现提议环境性因素而非遗传性或年龄相关因素触发这些疾病。有关报告表明慢性疾病发生率与和患病率美国过去20年巨量增加。全球各地看到类似增加。草甘膦除草剂1974年推出以来，抗除草剂转基因作物的出现推动草甘膦除草剂更大规模使用。越来越多证据表明草甘膦干扰农作物与动物中许多代谢过程，而且在农作物与动物体内检测到草甘膦残留。草甘膦干扰动物内分泌系统与肠道细菌平衡，损伤DNA并且说驱动导致癌症的突变。在该项研究中，在美国政府数据库搜索了转基因作物数据、草甘膦应用数据与疾病流行病学数据。对这些时间-序列数据组中22种疾病进行了相关性分析。草甘膦应用与一系列疾病之间的皮尔森相关系数高度显著：帕金森病（R = 0.875）。

Fig 26. Correlation between age=adjusted Parkinson's disease deaths and glyphosate application and percentage of US corn and soy crops that are

GE.

图26、对年龄进行统计调整帕金森病死亡率与草甘膦使用量和与美国转基因玉米、转基因大豆百分比之间相关性。

Nancy L. Swanson, Andre Leu, Jon Abrahamson and Bradley Wallet，Genetically engineered crops, glyphosate and the deterioration of health in the United States of America, Journal of Organic Systems，2014 Vol.9 No.2

Nancy L. Swanson, Andre Leu, Jon Abrahamson and Bradley Wallet，转基因作物、草甘膦以及美国国民健康恶化，有机系统杂志，2014 Vol.9 No.2

http://www.organic-systems.org/journal/92/abstracts/swanson-et-al.html

1. Abacus Enterprises, Lummi Island, WA, USA
2. International Federation of Organic Agricultural Movements, Bonn, Germany
3. Crustal Imaging Facility, Conoco Phillips School of Geology and Geophysics, University of Oklahoma, USA
1. 美国华盛顿州 Abacus Enterprises
2. 有机农业运动国际联合会，德国
3. 美国 Oklahoma 大学地质与地球物理学院

Figure 31. Age-adjusted incidence (AAIR) rates of thyroid cancer, by year and sex, for Central Valley, California 1988-2004.
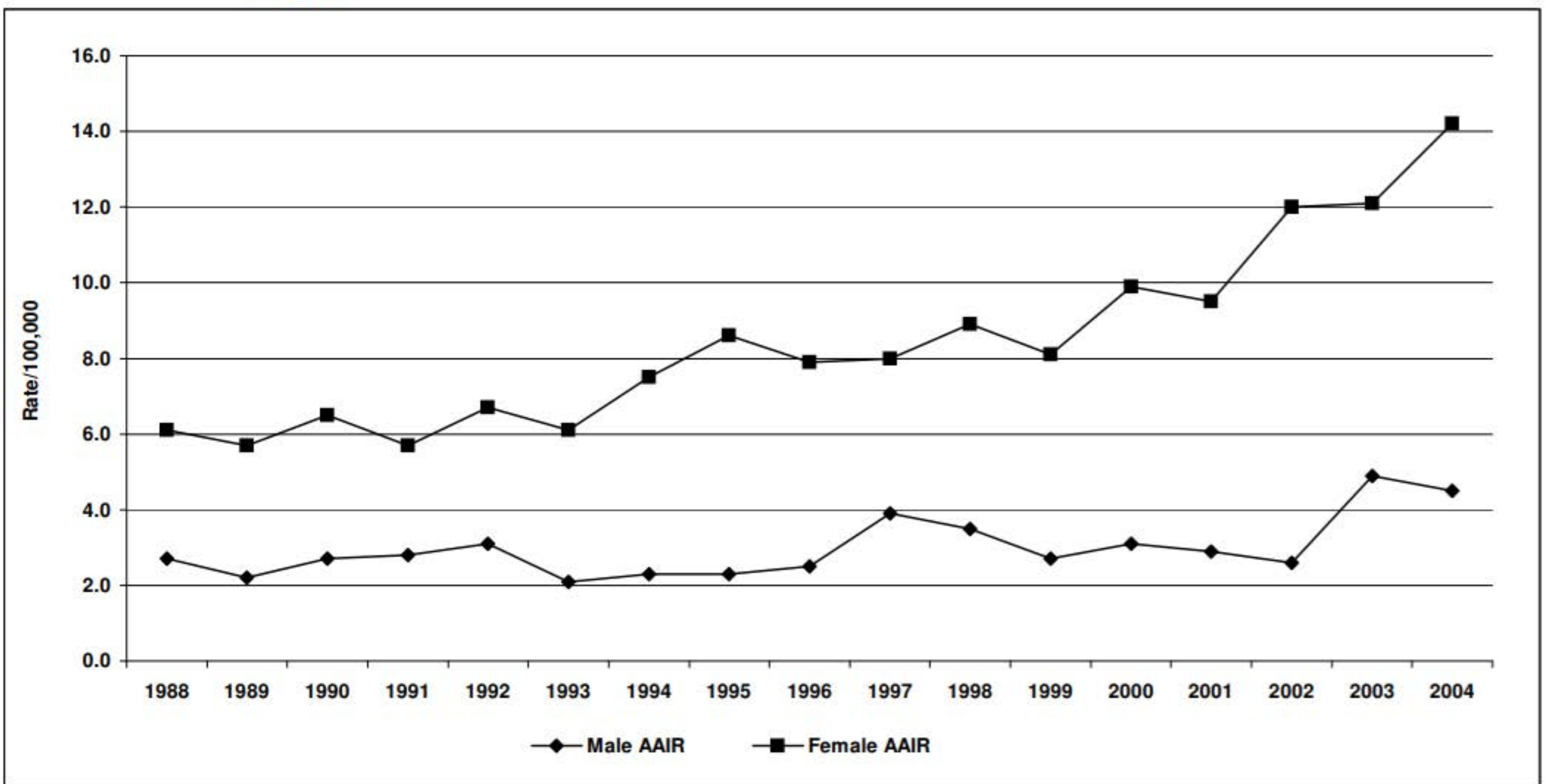
Incidence Rates† for California
Thyroid, 2009 - 2013
Hispanic (any race), Male, Ages <50
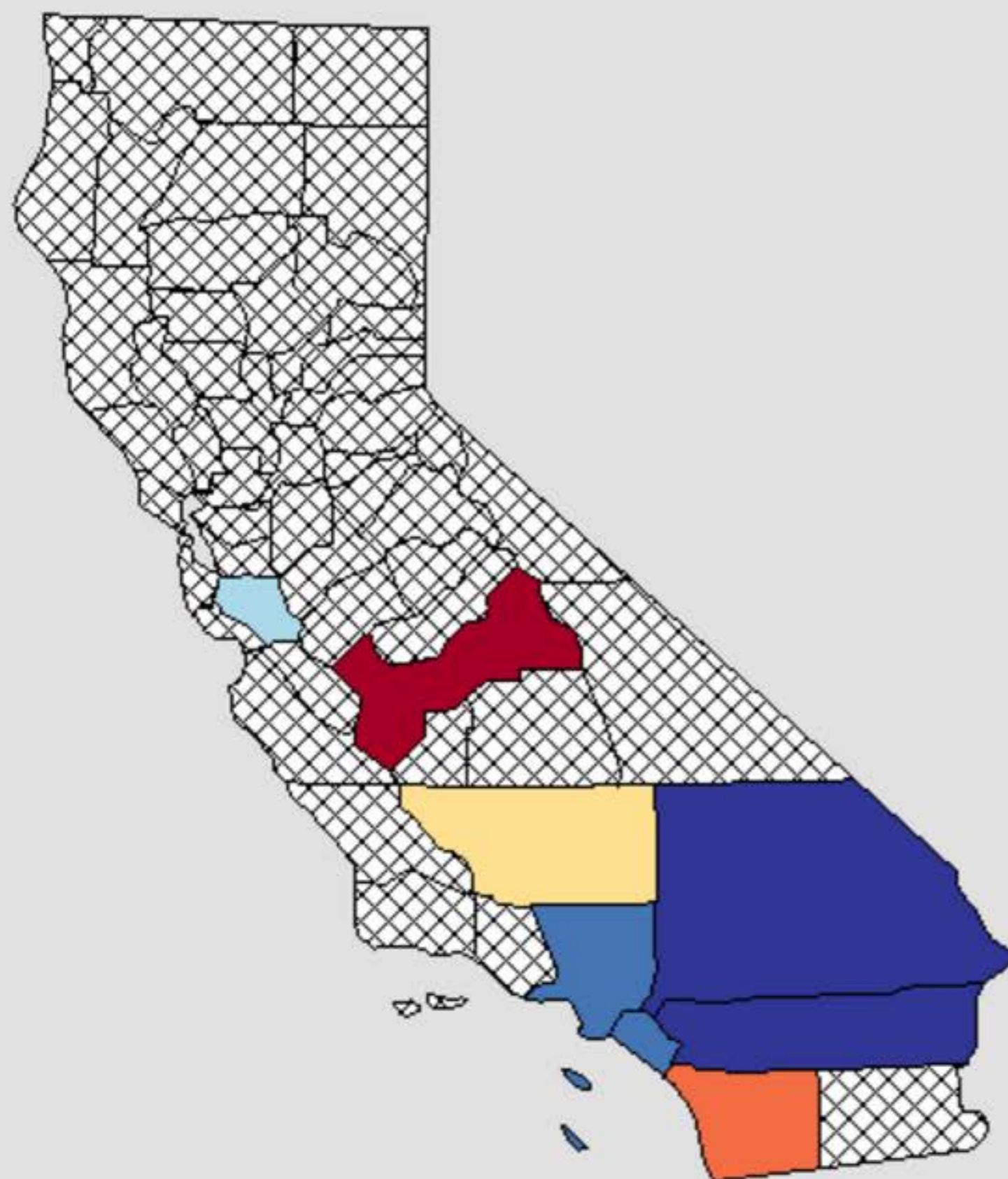
Age-Adjusted

Annual Incidence Rate

(Cases per 100,000)

Quantile Interval△

- 1.9 to 2.2
- 2.2 to 2.3
- 2.3 to 2.4
- 2.4 to 2.6
- 2.6 to 2.7
- 2.7 to 2.9
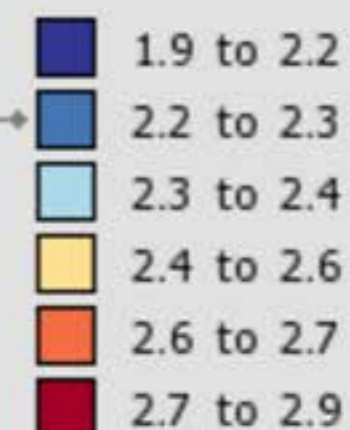
⊠ Suppressed * /**

US (SEER + NPCR)
Rate (95% C.I.)
Data not available.

California
Rate (95% C.I.)
2.3   (2.1 - 2.5)

Accepted Manuscript

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Please cite this article as: de Souza, Janaina Sena, Kizys, Marina Malta Letro, da Conceição, Rodrigo Rodrigues, Glebocki, Gabriel, Romano, Renata Marino, Ortiga-Carvalho, Tania Maria, Giannocco, Gisele, da Silva, Ismael Dale Cotrim Guerreiro, da Silva, Magnus Regios Dias, Romano, Marco Aurélio, Chiamolera, Maria Izabel, Perinatal exposure to glyphosate-based herbicide alters the thyrotrophic axis and causes thyroid hormone homeostasis imbalance in male rats.Toxicology http://dx.doi.org/10.1016/j.tox.2016.11.005

Perinatal exposure to glyphosate-based herbicide alters the thyrotrophic axis and causes thyroid hormone homeostasis imbalance in male rats

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1. Abstract

Glyphosate-based herbicides (GBHs) are widely used in agriculture. Recently, several animal and epidemiological studies have been conducted to understand the effects of these chemicals as an endocrine disruptor for the gonadal system. The aim of the present study was to determine whether GBHs could also disrupt the hypothalamic-pituitary-thyroid (HPT) axis. Female pregnant Wistar rats were exposed to a solution containing GBH Roundup®Transorb (Monsanto). The animals were divided into three groups (control, 5 mg/kg/day or 50 mg/kg/day) and exposed from gestation day 18 (GD18) to post-natal day 5 (PND5). Male offspring were euthanized at PND 90, and blood and tissues samples from the hypothalamus, pituitary, liver and heart were collected for hormonal evaluation (TSH – Thyroid stimulating hormone, T3 - triiodothyronine and T4 - thyroxine), metabolomic and mRNA analyses of genes related to thyroid hormone metabolism and function. The hormonal profiles showed decreased concentrations of TSH in the exposed groups, with no variation in the levels of the thyroid hormones (THs) T3 and T4 between the groups. Hypothalamus gene expression analysis of the exposed groups revealed a reduction in the expression of genes encoding deiodinases 2 (*Dio2)* and 3 (*Dio3)* and TH transporters *Slco1c1* (former *Oatp1c1*) and *Slc16a2* (former *Mct8*). In the pituitary, *Dio2*, thyroid hormone receptor genes (*Thra1* and *Thrb1),* and *Slc16a2* showed higher expression levels in the exposed groups than in the control group. Interestingly, *Tshb* gene expression did not show any difference in expression profile between the control and exposed groups. Liver *Thra1 and Thrb1* showed increased mRNA expression in both GBH-exposed groups, and in the heart, *Dio2*, *Mb*, *Myh6* (former *Mhca*) and *Slc2a4* (former *Glut4*) showed higher mRNA expression in the exposed groups. Additionally, correlation analysis between gene expression and metabolomic data showed similar alterations as detected in hypothyroid rats. Perinatal exposure to GBH in male rats modified the HPT set point, with lower levels of TSH likely reflecting post-translational events. Several genes regulated by TH or involved in TH metabolism and transport presented varying degrees of gene expression alteration that were probably programmed during intrauterine exposure to GBHs and reflects in peripheral metabolism. In conclusion, the role of GBH exposure in HPT axis disruption should be considered in populations exposed to this herbicide.

2. Introduction

To date, most studies concerning endocrine disruptors (EDs) have addressed the action of these compounds on the hypothalamic-pituitary-gonadal axis, showing varying degrees of interference (Bellingham et al. 2010; Rhind et al. 2010). However, only in recent years, studies have been published demonstrating interference in the hypothalamic-pituitary-thyroid (HPT) axis (Doerge and Chang 2002; Herbstman et al. 2008; Langer et al. 2009; Leung et al. 2010; Pearce and Braverman 2009; Pearce et al. 2010; Sathyapalan et al. 2011; Turyk et al. 2007), which is responsible for the precise secretion of thyroid hormones (TH) in the bloodstream.

THs are essential for the proper functioning of the body, taking part in various physiological processes, such as differentiation and tissue proliferation, training and maintaining the stability of the nervous system and metabolic balance. TH production is coordinated by the HPT axis, which is auto regulated by a negative feedback mechanism exerted by the active form of TH, triiodothyronine (T3). Hormonal action happens mainly through nuclear receptors (TH receptors isoform alfa and beta – THRa and THRb). The control of intracellular availability of T3 depends on the entrance of tetraiodothyronine (T4) into the cell using TH transporters (MCT8 and OATP1C1). Inside the cell, the T4 to T3 conversion is catalyzed by a 5′-deiodinase, that remove a molecule of iodine out of T4. There are three types of deiodinases (type I, II and III), these three enzymes are differently expressed in the various tissues, D1 and D2 are TH-activating enzymes, and D3 the inactivating one. Since thyroid produces mostly T4, the intracellular conversion mechanism is the most important source of peripheral T3 (Ortiga-Carvalho et al. 2016).

EDs are exogenous compounds with potential to alter hormonal regulation and the normal endocrine system (Casals-Casas and Desvergne 2011; Colborn et al. 1993; Vandenberg et al. 2012). This interference may occur in hormonal production, release and metabolism (Tabb and Blumberg 2006). Factors such as doses and period of exposure could interfere with the effects of this chemical in the endocrine system (Diamanti-Kandarakis et al. 2009; Schug et al. 2011; Vandenberg et al. 2012). Endocrine-disrupting agents comprise a wide variety of chemical classes, including pesticides, herbicides, detergents, repellents, flame-retardants and other compounds used in the plastics industry (Casals-Casas and Desvergne 2011; De Coster and van Larebeke 2012).

The studies reporting disruption of the HPT axis suggested alteration in various points of the HPT axis (Langer et al. 2009), such as thyroid hormones synthesis, action, peripheral concentration and thyroid hormone metabolism (Doerge and Chang 2002; Herbstman et al. 2008; Langer et al. 2009; Leung et al. 2010; Pearce and Braverman 2009; Pearce et al. 2010; Sathyapalan et al. 2011; Turyk et al. 2007). Some EDs have some definition about their mechanism of action, perchlorate is recognized and used in the past to treat thyrotoxicosis crisis for its antithyroidal effects, due to the inhibition of

iodine uptake by the sodium iodide symporter (NIS) (Tonacchera et al. 2004). More recently, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers or flame retardants (PBDEs) and Bisphenol A (BPA) were described with affinity with thyroid hormone receptor (THRs), acting as antagonist to T3 action (Boas et al. 2012; Freitas et al. 2011; Gilbert et al. 2012; Iwasaki et al. 2002; Kitamura et al. 2005a; Miyazaki et al. 2004; Moriyama et al. 2002; Sun et al. 2009). PCBs and PBDEs can also interact with the transthyretin (TTR) displacing T4 from this binding protein. Finally, pesticides, PCBs and PDBEs interfere with thyroid hormone hepatic clearance (Fini et al. 2007; Jagnytsch et al. 2006; Kitamura et al. 2005b).

Glyphosate-based herbicides (GBH) are one of the most studied EDs and have been used in agriculture for over 4 decades; however, in the last twenty years, with the introduction of genetically engineered glyphosate-tolerant crops, GBH application in agriculture has increased, and these compounds are currently the most used herbicide worldwide (Myers et al. 2016; Romano et al. 2012). Consequently, there is an increasing concern about the damage these compounds may cause to human health. Recently, several animal and epidemiological studies have contributed to this field of knowledge (Myers et al. 2016). Currently, GBHs are proposed as safe and non-toxic herbicides and were classified in 1991 as class E, non-carcinogenic substances (Pieniazek et al. 2003). The potential damages caused by these substances apparently depend on the applied concentration (Casals-Casas and Desvergne 2011; Marrs et al. 1989). However, even at a lower concentration than that recommended by the herbicide manufacturer and lower that the concentration detected in food, glyphosate is lethal to cells *in vitro* (Gasnier et al. 2009).

Glyphosate was already detected in the urine of children, mothers and fathers, in concentration up to 5.0 ng/ml in the urine of non farm individuals and up to 11 ng/ml in the urine of farm individuals, these results may be linked to the environment and also to exposure due to diet (Curwin et al. 2007). McQueen et al (2012) showed in their study the exposure of pregnant women to glyphosate is less than 2% of the acceptable daily intake and estimates that only 15% of this amount crosses the placenta (McQueen et al. 2012). The placenta is very important for intrauterine development controlling growth and substance exchange between the fetus and the mother (Aris and Leblanc 2011). Richard et al. (2005) have shown that in a nontoxic (bellow the recommended) of GBH was still toxic for human placental cell line and also it was disturbing to estrogen production (Richard et al. 2005). Benachour et al. (2007) showed study that GBH has a toxic and endocrine disrupting effect in human embryonic cells (in lower nontoxic concentration), in placental derived cells and fresh human placental cells was also sensitive to lower doses of GBH, showing endocrine disruptor characteristic of this substance, affecting human reproduction and fetal development (Benachour et al. 2007).

Furthermore, in 2015, the WHO International Agency for Research on Cancer reclassified GBHs as "probably carcinogenic to humans" (Guyton et al. 2015). In addition, studies in the gonadal axis have shown that glyphosate interferes with the

activity of aromatase, leading to changes in reproductive development in rats (Romano et al. 2012; Romano et al. 2010) and demonstrating the potential for endocrine disruption through GBH. However, there are few data available concerning the potential for GBH-mediated interference of the Hypothalamic-Pituitary-Thyroid (HPT) axis. In 2015, the Office of Pesticide Programs from the U.S. Environmental Protection Agency published a report from the Endocrine Disruptor Screening Program Tier 1 Assessment, that did not detected any evidence of glyphosate disrupting the thyroid pathway. This report was based in experiments with female and male pubertal assays, and amphibian metamorphosis, and used the glyphosate technical, not the commercial formulation (GBH) (Akerman and Blakinship, 2015).

Therefore, the aim of the present study was to verify the potential impact of a commercial GBH on the HPT axis to establish the risk for endocrine system disruption. Increasing awareness of this potential risk could stimulate more studies to establish plans for public health prevention and promotion.

3. Material and Methods

3.1 Animals, experimental design and treatment

Female and male Wistar rats (*Rattus norvegicus*) were mated in monogamous couples. For this study, 24 adult female were used (8 per group). Gestation day 1 (GD1) was confirmed by vaginal smear. Pregnant rats were treated by gavage with a suspension of Glyphosate Based Herbicide Roundup Transorb (Monsanto Co., St. Louis, MO; Monsanto of Brazil Ltd., São Paulo, Brazil) diluted in water from GD18 to post-natal day 5 (PND5). GBH doses of 5 and 50 mg/kg/day were selected based on the No Observed Adverse Effect Level (NOAEL) at 50 mg/kg/day for glyphosate, and the control group was similarly treated using deionized water (Romano et al. 2012; Romano et al. 2010). Litters were standardized to 8 pups per female (4 males and 4 females), totalizing 32 males per group of treatment. For the experiments of molecular biology, male rats of each litter were selected to represent all treated females.

The litters were maintained at eight male pups per female until PND21 (weaning). The male animals were subdivided into groups and maintained in polypropylene cages (43 x 43 x 20 cm) with a 5-cm layer of wood shavings. All animals were maintained on rat chow and water *ad libitum* under a 12:12 hour dark/light cycle in a temperature-controlled room (23 ± 1ºC). All procedures were performed in accordance with the Brazilian College of Animal Experimentation and were approved by the Bioethical Commission of Universidade Estadual do Centro-Oeste (UNICENTRO) (protocol number 509710316) and that of the Universidade Federal de São Paulo (UNIFESP) (protocol number 5097101316).

At PND90, the male animals were euthanized, and blood and tissues were collected for further analysis. The blood was collected via cardiac puncture to determine the TSH, T4 and T3 levels, and the tissue was excised for RNA extraction and real-time PCR assay analysis.

3.2 Hormone dosage

The TSH (thyroid stimulating hormone) serum concentration was determined using the Millipex Map Rat Kit, according to the manufacturer's recommendations. The T3 (triiodothyronine) and T4 (thyroxine) concentrations were measured through radioimmunoassay (ICN Pharmaceuticals, Costa Mesa, CA, USA) according to the manufacturer's protocol. For each dose, a standard curve was generated.

3.3 Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from the hypothalamus, pituitary, liver and heart using TRIzol® according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). To analyze the relative gene expression, quantitative real-time PCR was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Gene expression was determined using the $2^{\Delta\Delta Ct}$ method (Dussault and Pouliot 2006), and all values were expressed using cyclophilin A mRNA as a housekeeping, that did not present any variation between the studied tissues. The analyzed genes and their respective primers are presented in Table 1.

3.4 Metabolomic

In order to analyze the metabolomic profile four rat serum samples of each group were analyzed using targeted metabolomic approach of combined direct flow injection and liquid chromatography MS/MS using the AbsoluteIDQ p180 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria), this kit quantifies up to 188 endogenous metabolites from 5 different compound classes (i.e. acylcarnitines, amino acids, hexoses, phospho- and sphingolipids and biogenic amines). All analyzes were performed at the manufacturer's facility in Innsbruck, Austria. The sample concentration is expressed in micro Molar. Additionally, serum from 5 Wistar rats submitted to thyroidectomy used in another study already published by our group (da Conceicao et al. 2016), and 5 animals from the control group in the same study were used for the analysis. Hypothyroid animals were analyzed compared to their control group, GBH exposed animals were divided in high and low dose of exposition and compared to the respective control group. Another comparative study was done between the animals exposed to GBH divided in three or four groups based on ΔΔCt levels of the pituitary genes *Dio2* and *Slc16a2* (former *Mct8*).

3.5 Data analysis

The data related to hormone dosage and gene expression are reported as the means ±SEM values. The experiments were subjected to normality testing (Kolmogorov-Smirnov), followed by analysis of variance (one-way ANOVA) and Student-Newman-Keuls post hoc test when the results passed normality testing, and Kruskal-Wallis test and post hoc Dunn's Multiple Comparison Test when the results did not pass normality testing. Software Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used. Differences were considered significant at *P<0.05.*

The statistical analysis of the Metabolomic data was performed using the MetaboAnalyst 3.0 data analysis tool (www.metaboanalyst.ca) (Xia, J. 2015). Data was normalized by pooled control group and by generalized log transformation, and then submitted to Pearson correlation analysis, one-way ANOVA for data with more the two groups and t-test for experiments with two groups and post hoc Fisher's LSD, and unsupervised multivariated clustering analysis by heatmap.

4. Results

4.1 Clinical and hormonal features

Perinatal exposure to glyphosate did not alter the weight of the exposed animals compared with control. The groups exposed at 5 mg/kg/day (374.4 g ±4.05) and 50 mg/kg/day (377.4 g ±6.38) were similar in weight to the control group (365.9 ±4.93) (Table 2).

Thyroid hormone concentration analyses were performed in all three groups, and no alteration in the levels of T3 and T4 were observed between the groups exposed at 5 mg/kg/day (T3: 48.13 ng/dL ± 2.73; T4: 4.33 ng/dL ± 0.24) and 50 mg/kg/day (T3: 52.36 ng/dL ± 2.12; T4: 4.80 ng/dL ± 0.17) compared with the control (T3: 58.63 ng/dL ± 4.59; T4: 4.67 ng/dL ± 0.25). However, the TSH concentration significantly decreased in both groups exposed to glyphosate-based herbicides at 5 mg/kg/day (526.30 ng/dL ± 96.32) and 50 mg/kg/day (507.70 ng/dL ± 91.49) compared with control (962.50 ng/dL ± 152.10), as shown in Table 2.

4.2 Expression of deiodinases and thyroid hormone transporters decreased in the hypothalamus, resembling hypothyroidism in the pituitary

Hypothalamic genes associated with thyroid hormone metabolism showed altered expression in the animals exposed to glyphosate-based herbicides (Figure 1). *Dio2* expression decreased in animals exposed to 5 mg/kg/day (0.38 ±0.13) and 50 mg/kg/day (0.49 ±0.08) glyphosate-based herbicides compared with the control (1.08 ±0.16), and this difference was statically significant (p=0.006) (Figure 1 A). The same

effect was observed for *Dio3* gene expression: in animals exposed to glyphosate-based herbicides at 5 mg/kg/day (0.52 ±0.14) and 50 mg/kg/day (0.41 ±0.11) doses, the expression was lower compared with control animals (1.35 ±0.35) (p=0.0215) (Figure 1 B). The TH transporter gene *Slc16a2* (*Mct8*) presented decreased expression after exposure at 5 mg/kg/day (0.40 ±0.09) and 50 mg/kg/day (0.82 ±0.11) (p=0.0304) compared with the control (0.92 ±0.22) (Figure 1 C). *Slco1c1* (former *Oatp1c1*), another TH transporter, showed altered expression in both exposed groups at 5 mg/kg/day (0.48 ±0.13) and 50 mg/kg/day (0.59 ±0.12) compared with the control (1.24 ±0.16) (p=0.0012) (Figure 1 D).

Figure 2 shows the results of the gene expression analysis in the pituitary. *Tshb*, the gene responsible for the expression of the beta subunit of TSH, did not present any alterations in gene expression under GBH exposure (Figure 2 B). *Dio2* expression was increased in animal exposed at 5 mg/kg/day (1.79 ±0.30) and 50 mg/kg/day (1.05 ±0.21) (p=0.0217) compared with control (0.96 ±0.11) (Figure 2 A). In the lower dose group (5 mg/kg/day), the gene expression of the alpha 1 isoform of the TH receptor *Thra1* (1.78 ±0.33) and the beta 1 isoform of the TH receptor *Thrb1* (2.16 ±0.27) increased compared with the control (0.99 ±0.09 and 1.01 ±0.16, respectively), and similar results were obtained in the higher dose group (50 mg/kg/day; 0.77 ±0.06 and 1.07 ±0.24, respectively) (p=0.0019 *Thra1;* p=0.0025 *Thrb1*) (Figure 2 - C and D). The gene expression of *Slc16a2* increased in animals exposed at 50 mg/kg/day (2.18 ±0.12) compared with the control (1.02 ±0.11) and 5-mg/kg/day exposure (1.37 ±0.37) (p=0.0119) (Figure 2 E). *Slco1c1* expression was higher in animals exposed at 5 mg/kg/day (4.23 ±1.27) and 50 mg/kg/day (3.37 ±0.44) compared with the control (1.12 ±0.29) (p=0.0062) (Figure 2 F).

4.3 Peripheral T3-target systems are variably affected

In the liver, the genes associated with metabolism and hormone transport, *Dio1*, *Dio3* and *Slc16a2*, did not present any changes in gene expression in animals exposed to the herbicide. However, the expression of *Thra1* and *Thrb1* increased in the animals exposed at 50 mg/kg/day. The relative gene expression of *Thra1* was 1.78 ±0.14 in these animals compared with 1.03 ±0.08 in the control group (p=0.0085) (Figure 3 C). In addition, *Thrb1* also showed increased expression in animals exposed at 50 mg/kg/day (7.26 ±2.40) compared with the control (1.19 ±0.26) and 5 mg/kg/day exposure (2.21 ±0.90) (p=0.0303) (Figure 3 D).

Figure 4 shows the genes with altered expression under GBH exposure in heart tissue. *Dio2* gene expression decreased after exposure to herbicide at 50 mg/kg/day (0.22 ±0.11) compared with the control (1.68 ±0.68) (p=0.0424) (Figure 4 A). Cardiac alpha-myosin heavy chain (*Myh6* former *Mhca*), which is highly regulated by TH, showed increased expression increased at 50 mg/kg/day (54.82 ±20.56) compared with the control (2.57 ±1.84) and 5 mg/kg/day exposure (2.74 ±1.76) (p=0.0208) (Figure 4 B). Glucose transporter type 4 (*Slc2a4* former *Glut4*), a glucose transporter positively

regulated by TH, showed significantly decreased expression in animals exposed to 5 mg/kg/day (0.53 ±0.12) and 50 mg/kg/day (0.37 ±0.07) doses compared with the control (1.42 ±0.41) (p=0.0234) (Figure 4 C). Myoglobin (*Mb*), an important protein in muscles associated with oxygen storage and protection against reactive species of oxygen, showed decreased mRNA expression in animals exposed to 50 mg/kg/day herbicide (0.34 ±0.19) compared with the control (1.09 ±0.20) (p=0.0198) (Figure 4 D).

4.4 Correlation between gene expression and metabolite variation have a resemblance with the hypothyroid status

Correlation analysis of metabolite concentrations and the levels of *Dio2* and *Slc16a2* pituitary gene expression based in the ΔΔCt values resulted in a great number of positive and negative correlation (57 and 24, respectively, data not shown), in order to facilitate the analysis, the 10 most unsupervised correlated metabolites in each group were compared with hypothyroid and GBH exposed animals. There were concordances in part of the metabolites behavior with the same analysis made in animals with hypothyroidism and with the one done in animals exposed to GBH.

Dio2 gene expression had a positive correlation with the elevation of phosphatidylcholines and lysophosphatidylcholine, these elements are also elevated in both hypothyroid and GBH exposed rats comparing to control animals. (Figure 5a and 5b, table 3) A similar effect occurs with *Slc16a2* gene expression and phosphatidylcholine C32:3. For the *Slc16a2* gene expression, the amino acids leucine and valine, and the palmitoleylcarnitine decrease while the gene expression increase, and the low amino acid and acylcarnitine concentration is similar in hypothyroid and GBH exposed animals. (Figure 5c and 5d, table 3)

5. Discussion

The present study examined the disruption of the HPT axis of male adult rats by GBH perinatal exposure, resulting in reduced levels of TSH, the main hormone responsible for the stimulation of the production and release of TH. However, no significant difference in TH, T3 and T4 levels was detected in the same animals. This unusual hormone profile could reflect changes in TSH set-point resulting from altered gene programing in these animals during the fetal period, consistent with the results of other studies examining the effects of EDs, such as the flame retardant tetrabromo bisphenol A (TBBPA) and tributyltin (TBT), in the hypothalamus of mice. In animals exposed to these EDs, the transcription of genes controlled by TH, including hypothalamic *Trh*, was disrupted in the absence of T3, with a marked alteration of the hypothalamic set-point, which even interfered with metabolic responses (Decherf et al. 2010). However, unexpectedly, pituitary *Tshβ* mRNA levels did not change in the

exposed animals, although TSH peripheral levels decreased, likely reflecting an alteration in the post-translational processing of this protein (Goulart-Silva et al. 2011).

The analysis of the mRNA expression of genes associated with TH homeostasis was a good method to correlate changes in TH concentrations (Navarro-Martin et al. 2014), thus the present study focused on such genes, initially in the hypothalamus, which is likely the regulatory center of the hypothalamus-pituitary-thyroid (HPT) axis. The expression of the genes associated with TH homeostasis in the hypothalamus was compromised in exposed animals, and both deiodinase genes, *Dio2*, and *Dio3,* presented decreased expression. Deiodinases are enzymes responsible for thyroid hormone activation, the conversion of T4 to T3 (*Dio2*), or thyroid hormone deactivation, the conversion of T4 to reverse T3 (*Dio3*), and the synchronized work of both enzymes maintains normal TH concentrations inside the cell, as most of the T3 present in the cell is derived from the local conversion of T4 (Costa-e-Sousa and Hollenberg 2012). In tissues exposed to GBH, a decrease in both enzymes does not fit the regular model of TH regulation, as both activation and deactivation would be compromised. In addition, the hypothalamic expression of thyroid hormone transporters was also disrupted, with decreased *Slc16a2* and *Slco1c1* expression. TH transporters also play a key role in T_3 uptake in this gland, the disordered expression of *Slc16a2*, for example, suggests reduced T_3 for hypothalamic cells (Dumitrescu et al. 2006; Trajkovic et al. 2007). Thus, even if these animals presented normal peripheral TH concentrations, it is likely that the amount of T3 inside the hypothalamic cell could be compromised.

The main target of hypothalamic hormone stimulation is the pituitary, another key tissue associated with thyroid regulation (Ortiga-Carvalho et al. 2016). Herein, the gene expression of *Dio2, Thra1* and *Thrb1* increased only in animals exposed to a lower dose of GBH, and *Slc16a2* mRNA expression was also increased, but only at the higher GBH dose, and *Slco1c1* expression was increased at both doses. Apparently, the pituitary is more sensitive to a lower concentration of glyphosate than a higher concentration, which is not unusual for the EDs, as these compounds do not necessarily follow typical hormone dose-response curves (Lagarde et al. 2015). Similar to hormones, EDs operate at relatively low doses in a tissue-specific manner and may also exhibit traditional dose-response effects not observed with other drugs, reflecting the complicated dynamics of receptor occupation and saturation. Thus, lower doses may be more deleterious than higher doses, completely changing the dose effect curve response of a certain receptor (Diamanti-Kandarakis et al. 2009; Schug et al. 2011; Vandenberg et al. 2012). In addition, these gene expression profiles resemble a hypothyroid state.

To investigate the possible disruptive role of GBH in TH action and metabolism in peripheral tissues, liver and heart samples were collected. These two organs are the primary targets for TH action and could be models for this evaluation. Liver is one of the most important targets of TH and is also a fundamental organ in metabolism, particularly the control of TH homeostasis (Ortiga-Carvalho et al. 2016). In contrast to the disruptions observed in the gene expression of central hypothalamic-pituitary

deiodinases and TH transporters, in the liver there was no change in the mRNA expression of these genes under GBH exposure. Nevertheless, the mRNA expression of TH receptors increased after exposure, indicating that the response to TH in the liver may decrease under GBH exposure, despite the normal hormone serum concentration. In addition, *Thrb1* is an important TH receptor in the liver for lipid metabolism, and this gene could potentially be compromised in this situation (Pramfalk et al. 2011).

Another important tissue that is target for TH action is the heart, the most important organ associated with cardiovascular homeostasis and adaptation to hemodynamic variation, an impaired heart function could lead to pulmonary and systemic congestion and even death (Neves et al. 2015). Thus, maintaining the right level of TH in this tissue is critical, GBH exposure decreased mRNA levels of *Dio2*, indicating a decrease in T4 to T3 conversion. However, in this tissue, GBH acted ambiguously with respect to TH activity, while *Dio2* mRNA expression decreased and *Mhca* increased, a response expected under conditions of high TH levels (Danzi et al. 2008). In cardiac muscle, thyroid hormone increases *Myh6* expression, associated with the increased speed of muscle contraction and relaxation; however, hypothyroidism makes this muscle achieve a hypokinetic state (Danzi and Klein 2004). In contrast, *Slc2a4* and *Mb* mRNA expression decreased at a higher GBH concentration, inconsistent with the TH regulation pattern. Giannocco et al (Giannocco et al. 2004) showed that TH increases *Mb* gene and protein expression to protect tissues against damage from excess hormone. TH also stimulates *Slc2a4* gene expression, a glucose transporter pivotal for cardiac metabolism (Gosteli-Peter et al. 1996; Weinstein and Haber 1992). The decreased mRNA expression of *Slc2a4* under GBH exposure may indicate a decrease in glucose uptake and oxygen supply and a subsequent decrease in cell metabolism, although cardiac muscle activity should increase, as evidenced by the increase in *Myh6* gene expression. In addition, another TH-regulated gene, *Myh7* (former *Mhcb*), did not present any changes in gene expression in the present study (data not shown), although previous studies have shown that this gene is negatively regulated by TH (Danzi and Klein 2004). Pazos-Moura and colleagues (Pazos-Moura et al. 2000) showed that an overexpression of a mutant TH receptors in heart did not affect the expression of *Myh6* or *Myh7* in either hypothyroidism or hyperthyroidism, likely indicating that TH receptors are essential to the effects of TH in these genes. This finding is reinforced by a study with mice with the Δ337T mutation in *Thrb* gene that also showed the important role of this isoform in the regulation of *Myh6 and Myh7* mRNA expression in heart (do Imperio et al. 2015).

The correlation between pituitary gene expression regulation and the metabolic profile of the GBH exposed animals and peripheral metabolic alteration showed a partial resemblance with hypothyroidism, what is probably not surprising, due to multivariate influence of GBH in the metabolism. The association between the gene expression, GBH exposition and the differences in the serum metabolites were similar with a study performed with the same method in human serum of euthyroid subjects. This study showed a positive association between free T4 levels and acylcarnitine

(particularly C16:1 the same present in association with *Slc16a2* gene expression), and a negative association with the group of phosphatidylcholines compounds (PC acyl – aa, PC acyl-akyl – ae, and Lyso PC acyl – a), this study showed that even small changes in thyroid hormone, between the normal range, could promote important changes in metabolite concentrations (Jourdan et al. 2014).

Previously, the Endocrine Disruptor Screening Program Tier 1 Assessment from de US EPA did not detected any evidence of disruption in the thyroid pathway. The study was based on pubertal assays for male and female rats, and frog metamorphosis. It analyzed hormonal dosage, development stages and physical characteristics; however, the study did not evaluate gene expression in the HPT axis, neither metabolomic profile. Another important difference with the present study is the type of chemical used, the EPA study used isolated glyphosate, and our study was performed with a commercial GBH, that includes adjuvants such as surfactants, known to make the GBH more toxic than glyphosate alone (Folmar et al. 1979; Mesnage et al. 2013; Tsui and Chu 2003). The adjuvants are important to glyphosate adhesion and translocation into to the sub-surface plant tissues, and they are also present as residues in the plants together with glyphosate itself, what could possibly facilitate the contamination since the population exposition is to the commercial chemical set and not only the glyphosate (Myers et al. 2016).

The other important difference between the two studies is the window of exposure, the present study showed that the time of exposition is important, and early embryonic development exposure could have different impact than adulthood exposure. Several studies have shown that exposure to EDs, even at low doses, during the embryonic period may have persistent effects even after the end of exposure, suggesting that EDs are the basis for diseases presented only in adulthood (Diamanti-Kandarakis et al. 2009; Schug et al. 2011). The data in the present study suggested that perinatal exposure to GBH generated persistent alterations in several aspects of TH homeostasis, even at adulthood.

In summary, the present study showed that GBH exposure during the perinatal period in male rats disrupts the TSH set-point, likely reflecting a post-translational process, and also disrupts the expression of several genes associated with thyroid hormone homeostasis and function (Figure 6), without altering the peripheral hormonal concentration, but alters the metabolomic profile, similar to a hypothyroid state. Overall, these results suggest that changes in the programming of the HPT axis may occur after GBH exposure. However, more studies, particularly epidemiological studies, are needed to clarify the precise effect of this ED on the HPT axis and develop strategies for public health actions.

Acknowledgement

6. **Tables and Figures Legends**

Table 1. List of genes with respective primer sequences analyzed and presented in this study

Table 2. Animal weight and hormonal dosage (TSH, T3 and T4)

The animals were weighed prior to blood collection. Blood was collected through cardiac puncture, and following centrifugation, the serum was stored at -20°C until further analysis. The data are shown as the means±SEM values. For weight, the Kruskal-Wallis test and Dunn's posttest were applied. For TSH, T3 and T4, one-way ANOVA and the Student-Newman-Keuls post hoc test were used. A total of 7-8 animal were included per group.

[*]P<0.05 compared with control

Table 3. p-value from t-student test and ANOVA from the correlation between metabolomic analysis: hypothyroid rats vs. control, gene expression variation (*Mct8 - Slc16a2* and *Dio2*) animals exposed do glyphosate vs. control and GBH exposed animals control vs. high vs. low dose.

PC - phosphatidylcholines compounds (PC acyl – aa, PC acyl-akyl – ae, and Lyso PC acyl – a). C16:1 – acylcarnitine 16:1, palmitoleylcarnitine. Ile – amino acid isoleucine, Leu – amino acid leucine, Val – amino acid valine, His – amino acid histidine, Xle – amino acid – sum of Ile and Leu. SM C24:0 – Sphingomyelin C24:0.

Figure 1. Effect of perinatal exposure to glyphosate on the gene expression of proteins associated with thyroid hormone homeostasis in the hypothalamus. (A) *Dio2* showed decreased gene expression after treatment at both 5 and 50 mg/kg/day compared with the control; (B) *Dio3* showed decreased gene expression after treatment at both 5 and 50 mg/kg/day compared with the control; (C) At 5 mg/kg/day, thyroid hormone transporter *Mct8* (*Slc16a2)* showed decreased gene expression compared with the control and 50 mg/kg/day treatment; (D) thyroid hormone transporter *Oatp1c1* (*Slco1c1*) showed decreased expression at both 5 and 50 mg/kg/day, compared with the control. **P=0.006 and *P<0.05. The data are presented as shown as the means ±SEM values. A and D Kruskal-Wallis test and Dunn's Multiple Comparison Test. B and C one-way ANOVA and Newman-Keuls Multiple Comparison Test.

Figure 2. Graphic representation of gene expression of protein associated with thyroid hormone homeostasis in pituitary of rats after perinatal treatment with glyphosate. (A) Significant increasing of *Dio2* in group 5 mg/kg/day compared with control and 50 mg/kg/day; (B) The expression of *Tshb* suffer no alteration in exposed animals; (C) Thyroid hormone receptor isoform alpha1, *Thrα1,* had a significant increase in expression at group 5 mg/kg/day compared with control and 50 mg/kg/day; (D) Thyroid hormone receptor isoform beta 1, *Thrβ1,* has its expression increased at group 5

mg/kg/day compared with control and 50 mg/kg/day; (E) Thyroid hormone transporter *Mct8* (*Slc16a2*) has an increasing in the expression of group 50 mg/kg/day compared with control; (F) *Oatp1c1* (*Slco1c1*) has an increase of expression at groups 5 mg/kg/day and 50 mg/kg/day compared with control. **P=0.002 and *P<0.05. Data shown are means ±SEM values. B and F Kruskal-Wallis test, Dunn's Multiple Comparison Test. A, C, D and E One-way ANOVA, Newman-Keuls Multiple Comparison Test.

Figure 3. Graphic representation of gene expression of protein associated with TH homeostasis in livers of animals treated with glyphosate during the perinatal period. (A-B-E) *Dio1*, *Dio3* and *Mct8* (*Slc16a2)* expression are not altered in treatments compared with the control; (C-D) the expression of TH receptors *Thrα1* and *Thrβ1* had the expression in group 50 mg/kg/day increased compared with control (*Thrα1* and *Thrβ1*) and compared with 5 mg/kg/day (*Thrβ1*). **P=0.0085 and *P<0.05. The data are shown as the means ±SEM values. B and C Kruskal-Wallis test and Dunn's Multiple Comparison Test. A, D and E One-way ANOVA, Newman-Keuls Multiple Comparison Test.

Figure 4. Gene expression of proteins associated with TH homeostasis and responsiveness in the heart. (A) *Dio2* showed decreased expression at 50 mg/kg/day and 5 mg/kg/day compared with control; (B) *Mhcα* (*Myh6*) gene expression at 50 mg/kg/day was higher when compared with control and 5 mg/kg/day; (C) The expression of *Glut4* (*Slc2a4*) in both treatment groups decreased compared with the control; (D) *Mb*, a muscle globin, showed decreased expression at 50 mg/kg/day compared with control and 5 mg/kg/day. *P<0.05. The data are shown as the means ±SEM values. A and B Kruskal-Wallis test and Dunn's Multiple Comparison Test. C and D One-way ANOVA and Newman-Keuls Multiple Comparison Test.

Figure 5. Metabolomic analysis of animals exposed to GBH and hypothyroidism. (A) Heatmap unsupervised clustering analysis showing the 10 metabolites with stronger association between their concentration and levels of *Dio2* gene expression. The groups are represented in colors in the top: red for ΔΔCt values from 0.8 to 1.3, green values from 1.4 to 1.7 and blue from 1.8 to 2.6. The positive or negative correlation follows the color scheme as indicated. (B) Graphs representing metabolite normalized concentration, graph Hypo represents the metabolite in hypothyroid animals compared to control, graph Dio2 is divided in three groups following *Dio2* gene expression based in ΔΔCt values, and graph Glyphosate showed the normalized concentration of the metabolite in the control group and in the animals exposed to the high and low dose of GBH as indicated. (C) Heatmap unsupervised clustering analysis showing the 10 metabolites with stronger association between their concentration and levels of *Mct8* (*Slc16a2)* gene expression. The groups are represented in colors in the top: red for ΔΔCt values from 0.4 to 0.9, green values from 1.0 to 1.3, blue from 1.4 to 2.0 and light blue from 2.1-2.5. The positive or negative correlation follows the color scheme as indicated. (D) Graphs representing metabolite normalized concentration, graph Hypo represents

the metabolite in hypothyroid animals compared to control, graph Mct8 is divided in four groups following *Mct8 (Slc16a2)* gene expression ΔΔCt values, and graph Glyphosate showed the normalized concentration of the metabolite in the control group and in the animals exposed to the high and low dose of GBH as indicated. The p-values for all the metabolites are reported in table 3.

PC - phosphatidylcholines compounds (PC acyl – aa, PC acyl-akyl – ae, and Lyso PC acyl – a). C16:1 – acylcarnitine 16:1, palmitoleylcarnitine. Ile – amino acid isoleucine, Leu – amino acid leucine, Val – amino acid valine, His – amino acid histidine, Xle – amino acid – sum of Ile and Leu. SM C24:0 – Sphingomyelin C24:0.

Figure 6. Summary of the effects of GBH on thyroid hormone metabolism, the peripheral hormonal concentrations, and gene expression of proteins associated with thyroid hormone metabolism and action in the hypothalamus, pituitary, liver and heart. *Mct8 - Slc16a2, Oatp1c1 - Slco1c1, Mhcα - Myh6, Glut4 - Slc2a4*.

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Tables

Table 1. List of genes with respective primer sequences analyzed and presented in this study

Gene	Forward	Reverse
Tshβ	5'-CAGCATTAACTCGCCAGTGC-3'	5'-AAGCAAGAGCGAAAAGCACG-3'
Thrβ1	5'-TGGGCGAGCTCTATATTCCA-3'	5'-ACAGGTGATGCAGCGATAGT-3'
Dio1	5'- GCCATTCCCCTGCTGTAACT - 3'	5'- CCGTCAGTCCAAAGCCATCT – 3'
Dio2	5'-AGAAGCACCGGAACCAAGAG-3'	5'-AGCCACAACTTGACACTGGG-3'
Dio3	5'- GCCTCTACGTCATCCAGAGC - 3'	5'- GCCCACCAATTCAGTCACTT – 3'
Cyclophilin A	5'-GTCAACCCCACCGTGTTCTTC-3'	5'-ACTTGCCACCAGTGCCATTATG-3'
Slc16a2/Mct8	5'-CCCAAGCAAGAGAGGCGCCC-3'	5'-CGGTAGGTGCGCTGGCGAAA-3'
Slco1c1/Oatp1c1	5'-GGATCCCCAGTGGGTCGGGG-3'	5'-ACCAGAAAGGCACGGCTGCA-3'
Myh6/Mhcα	5'-ACAAGGTTAAAAACCTGACAGAGG-3'	5'-TACTGTTCTGCTGACTGATGTCAA-3'
Slc2a4/Glut4	5'-CCGCCAGGCCGGGACACTAT-3'	5'-TCCGTCGGAAGGTGGCCGAG-3'
Mb	5'-CCGGTCAAGTACCTGGAGTT-3'	5'-TGAGCATCTGCTCCAAAGTC-3'
Thrα1	5'- ACCTCCGCATGATCGGGGC – 3'	5'- CCTGATCCTCAAAGACCTC – 3'

Table 2. Animal weight and hormonal dosage (TSH, T3 and T4)

	Control	5mg/kg/day	50mg/kg/day
Animal weight (g)	365.9 ± 4.933	374.4 ± 4.053	377.4 ± 6.383
TSH (ng/dL)	962.5 ± 152.1	$526.3 \pm 96.32^{*}$	$507.7 \pm 91.49^{*}$
T3 (ng/dL)	58.63 ± 4.590	48.13 ± 2.730	52.36 ± 2.123
T4 (ng/dL)	4.674 ± 0.2476	4.335 ± 0.2404	4.801 ± 0.1748

The animals were weighed prior to blood collection. Blood was collected through cardiac puncture, and following centrifugation, the serum was stored at -20°C until further analysis. The data are shown as the means±SEM values. For weight, the Kruskal-Wallis test and Dunn's posttest were applied. For TSH, T3 and T4, one-way ANOVA and the Student-Newman-Keuls post hoc test were used. A total of 7-8 animal were included per group.

*P<0.05 compared with control

Table 3. p-value from t-student test and ANOVA from the correlation between metabolomic analysis.

Note 1 – Hypothyroidism - hypothyroid rats vs. control, Mct8 and Dio2 - gene expression variation (*Mct8 - Slc16a2* and *Dio2*) animals exposed do glyphosate vs. control, and Glyphosate - control vs. GBH exposed animals high vs. low dose.

Hypothyroidism		Mct8		Dio2		Glyphosate	
Metabolite	p-value	Metabolite	p-value	Metabolite	p-value	Metabolite	p-value
Val	0.0005	Val	0.00854	Val	-	Val	0.0010777
Leu	0.0141	Leu	0.0075641	Leu	-	Leu	0.024874
C16.1	0.0257	C16.1	0.0018079	C16.1	-	C16.1	0.56607
PC aa C32.3	0.0028514	PC aa C32.3	0.0098788	PC aa C32.3	0.00073439	PC aa C32.3	0.0083428
Pc ae C44.4	0.13732	Pc ae C44.4	0.0080159	Pc ae C44.4	0.00071489	Pc ae C44.4	0.0027811
PC aa C24.0	0.0112	PC aa C24.0	-	PC aa C24.0	0.0010062	PC aa C24.0	0.032443
LysoPC a C 28.0	0.00026958	LysoPC a C 28.0	-	LysoPC a C 28.0	0.0017758	LysoPC a C 28.0	0.034861
PC aa C42.1	0.0031769	PC aa C42.1	-	PC aa C42.1	0.0015331	PC aa C42.1	0.048676
PC aa C42.0	0.024813	PC aa C42.0	-	PC aa C42.0	0.0015331	PC aa C42.0	0.17802
PC ae C44.5	0.049346	PC ae C44.5	-	PC ae C44.5	0.00081893	PC ae C44.5	0.0037234

Note 2 - PC - phosphatidylcholines compounds (PC acyl – aa, PC acyl-akyl – ae, and Lyso PC acyl – a). C16:1 – acylcarnitine 16:1, palmitoleylcarnitine. Ile – amino acid isoleucine, Leu – amino acid leucine, Val – amino acid valine, His – amino acid histidine, Xle – amino acid – sum of Ile and Leu. SM C24:0 – Sphingomyelin C24:0.
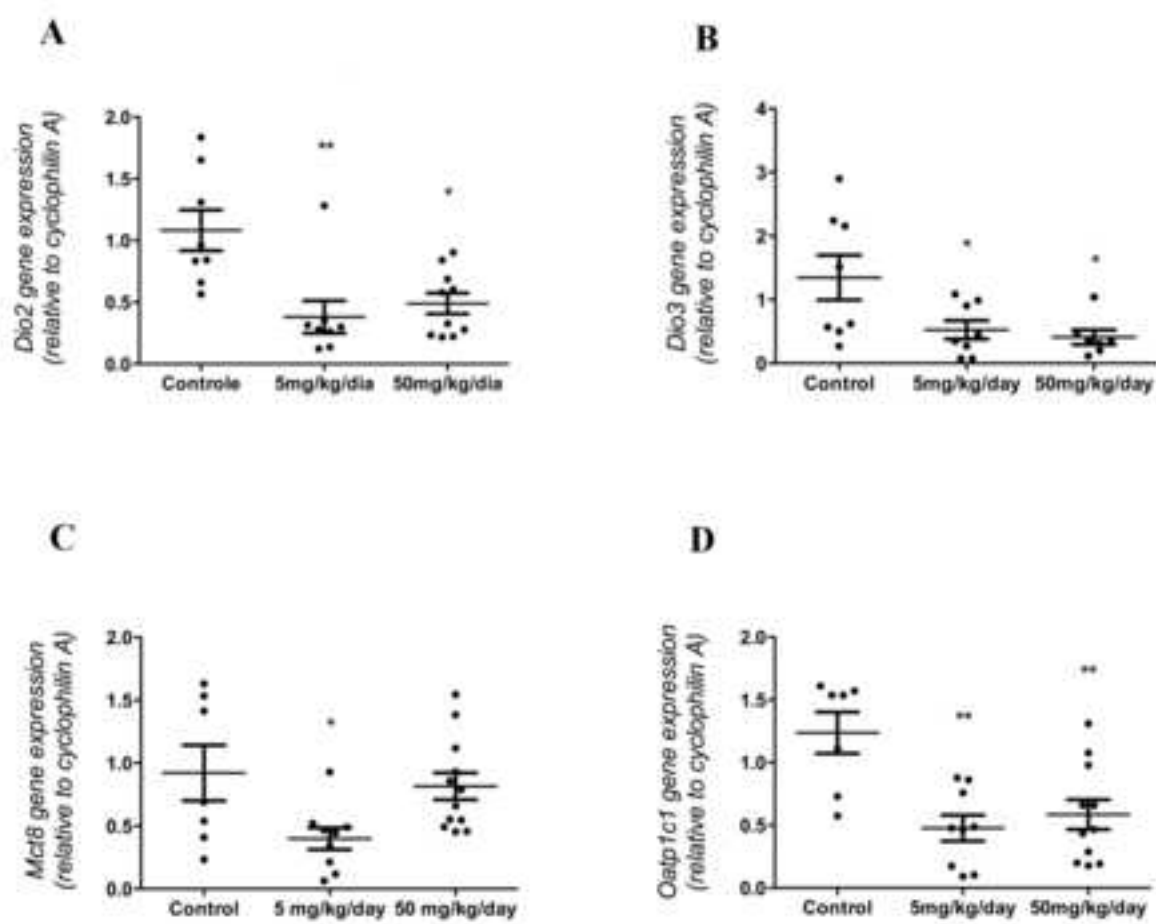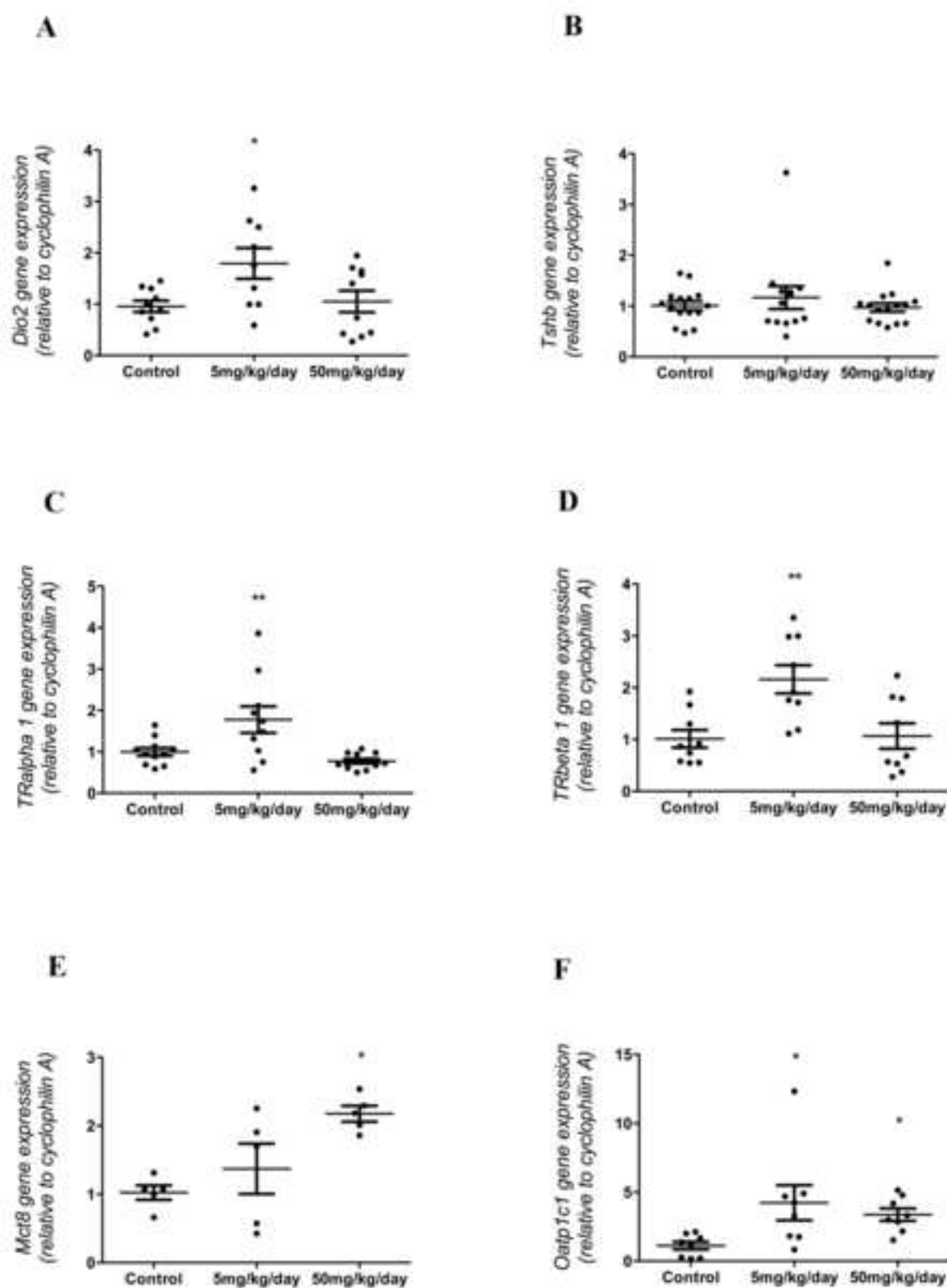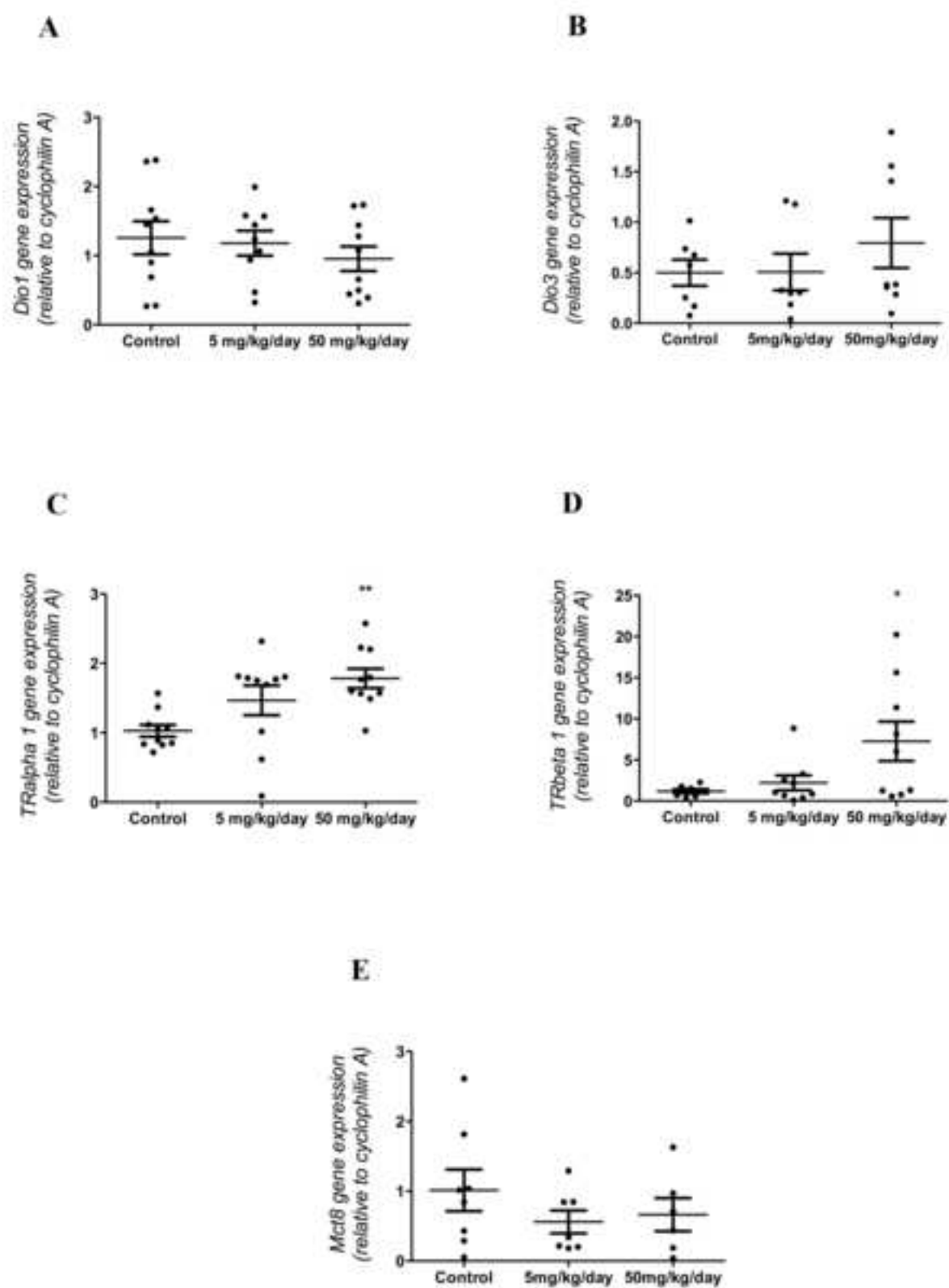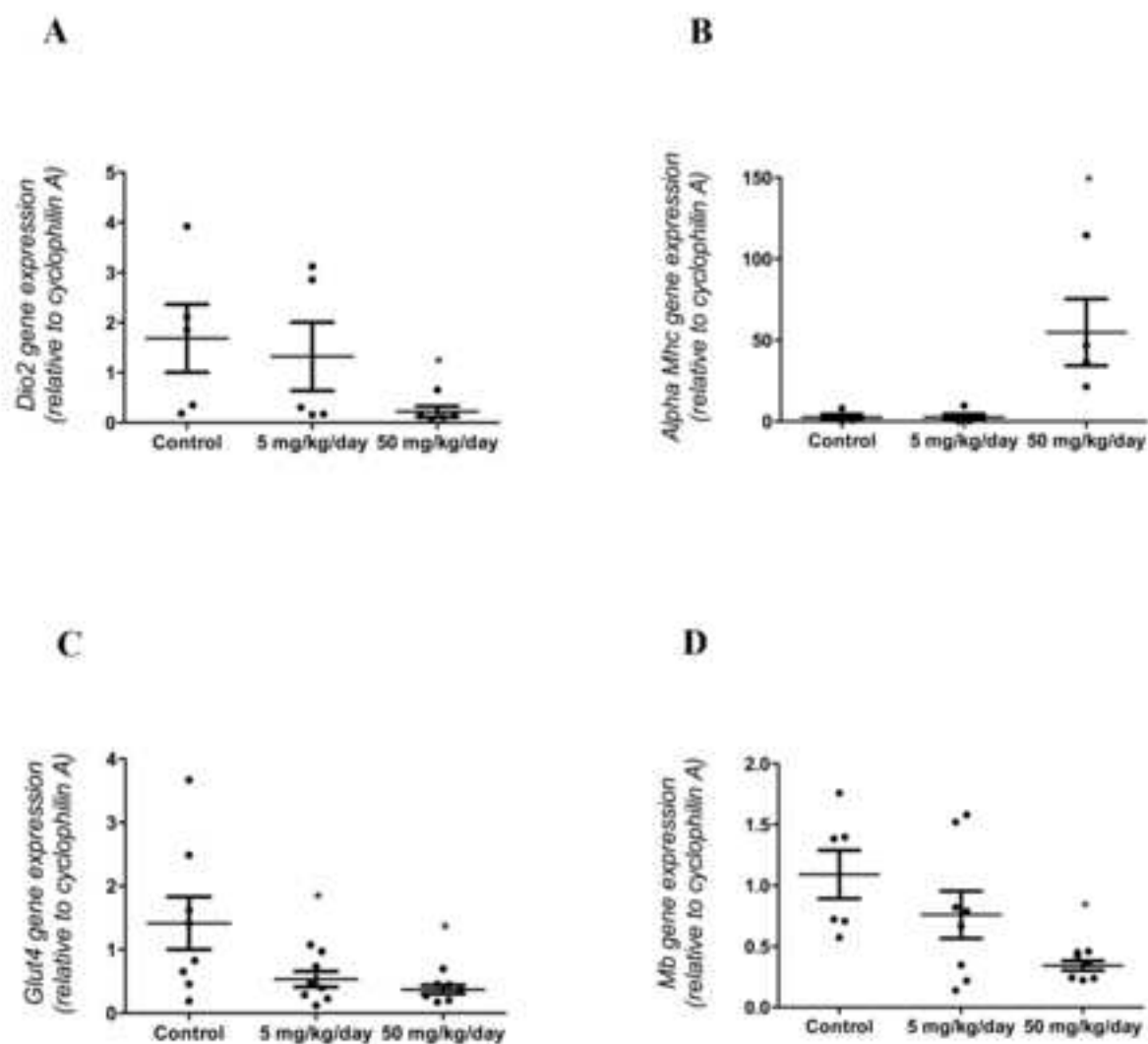
Figure 1 ACCEPTED MANUSCRIPT

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

ACCEPTED MANUSCRIPT

A

B

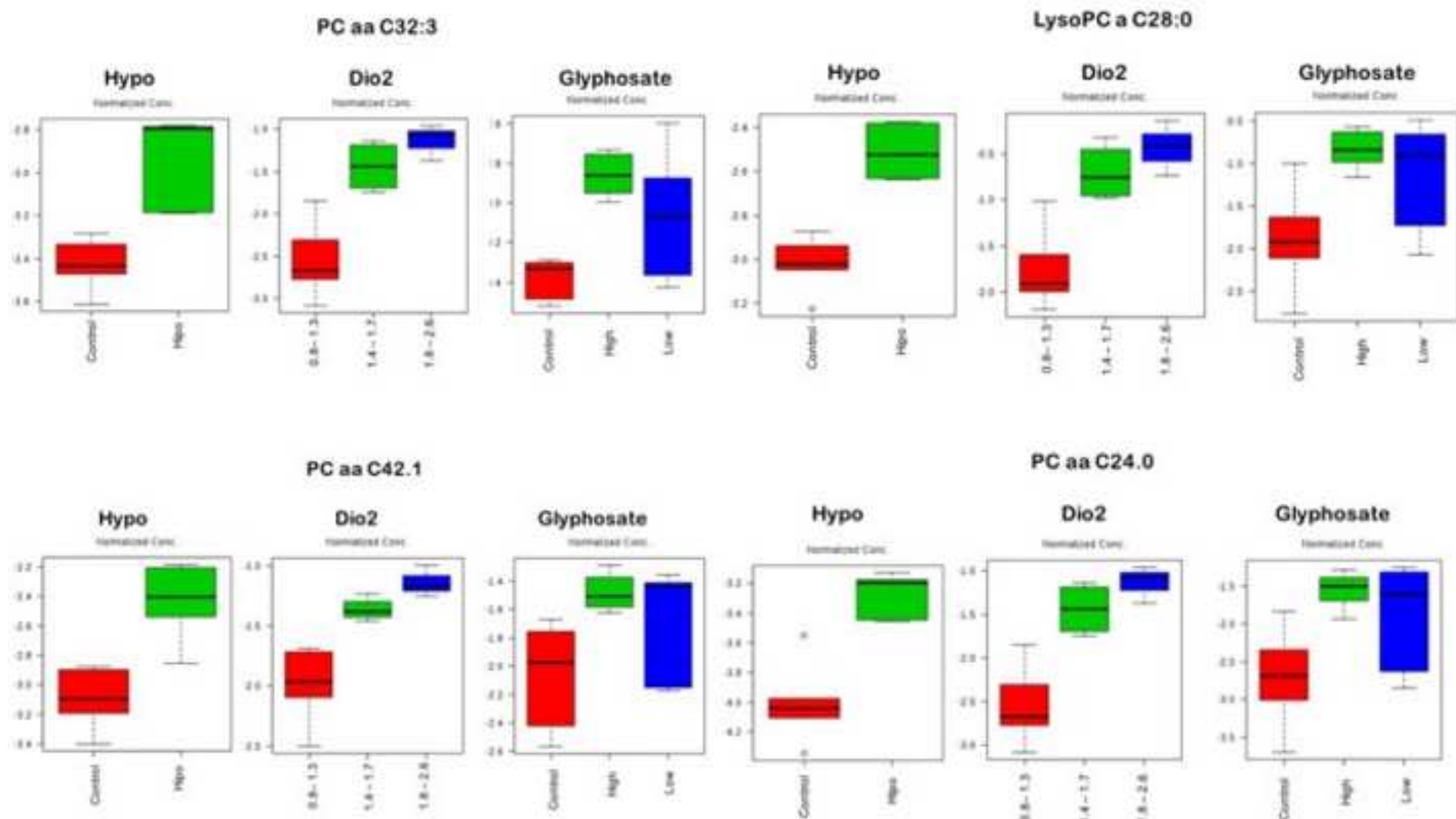**C**

D

Figure 4

Metabolomic Correlation with pituitary Dio2
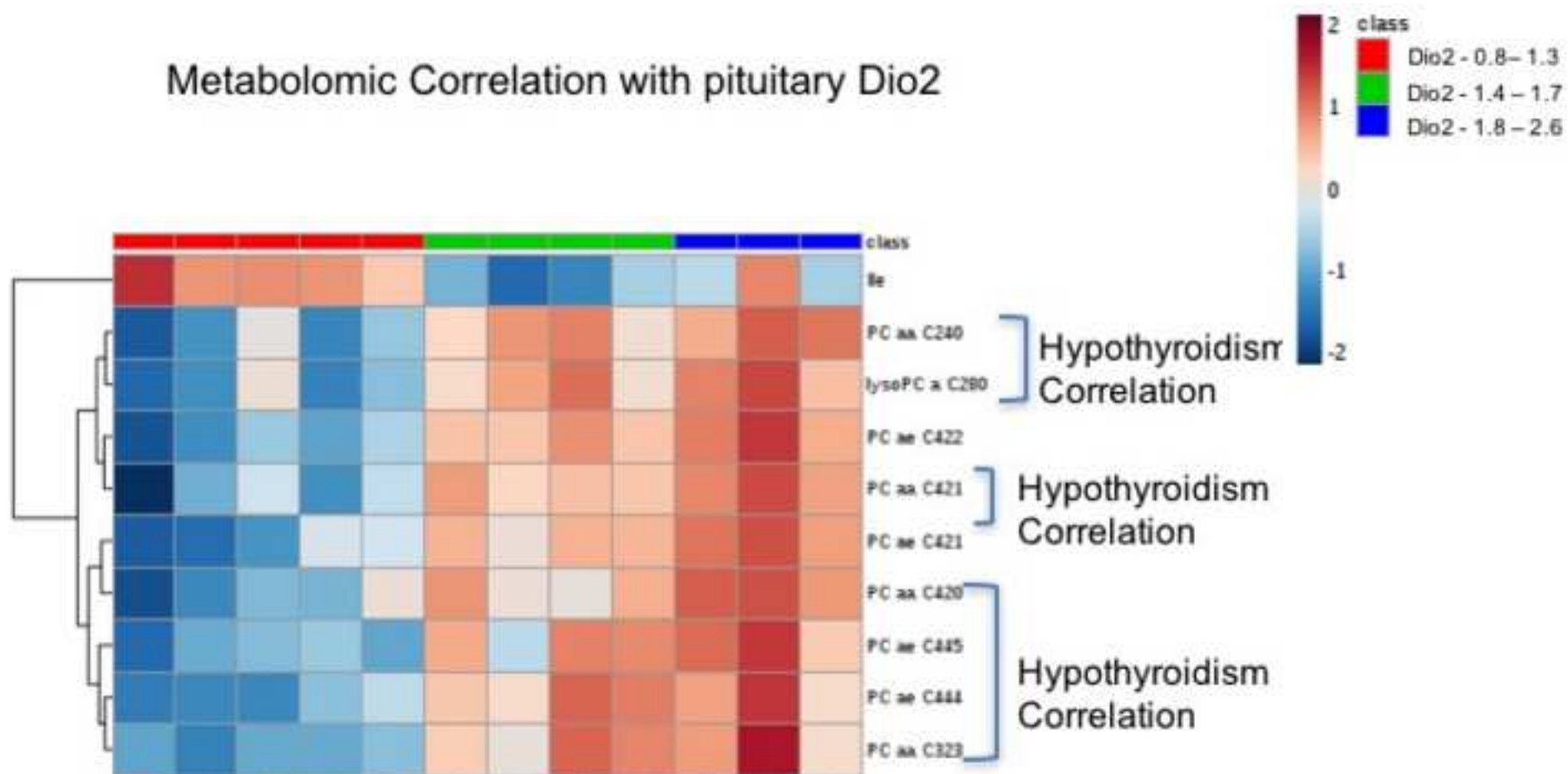
Metabolomic Correlation with pituitary Mct8

Figure 5D

Figure 6